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UNIVERSITY OF ALBERTA

Production, Purification and Characterization of a *Leuconostoc* Bacteriocin and Analysis of its Genetic Determinants

by

John W. Hastings

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

IN

Food Microbiology

Department of Food Science

EDMONTON, ALBERTA

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To Becky

Abstract

A heterofermentative lactic acid bacterium, strain UAL 187, isolated from meat packaged under elevated CO_2 levels was identified as *Leuconostoc gelidum*. It grows well at refrigeration temperatures but not at 35°C. Initial studies showed that this organism produces an inhibitory substance that is inactivated by Pronase E and trypsin, but not by catalase or by heating at 62°C for 30 min. This inhibitory substance, leucocin A-UAL 187, is produced early in the growth cycle, at 1, 5 and 25°C. The inhibitory substance is active against a large number of closely related lactic acid bacteria, as well as a strain of *Enterococcus faecalis* and *Listeria monocytogenes*.

The bacteriocin was purified by ammonium sulphate or acid (pH 2.5) precipitation, hydrophobic interaction chromatography, gel filtration and RP-HPLC with a yield of 58% of the original activity. Leucocin A-UAL 187 is stable at low pH, heat resistant and the pure form is stabilized by the addition of bovine serum albumin. It is inactivated by a range of proteolytic enzymes. The molecular weight was determined by mass spectrometry as 3930.4 ± 0.2. DNA sequencing revealed that leucocin A-UAL 187 contains 37 amino acids with a calculated molecular weight of 3932.3. From this a disulphide bridge between the two cysteines is postulated. However, destroying the disulphide bridge does not destroy the activity of leucocin A-UAL 187.

Initial evidence suggested that the genetic information determining production of, and resistance to, the bacteriocin is plasmid mediated. Of the three plasmids found in this organism, loss of the 7.6 MDa plasmid resulted in loss of production of and immunity to the bacteriocin. Loss of the 5.0 MDa plasmid did not result in a detectable phenotypic change in the organism.

The first thirteen amino acids at the N-terminus of leucocin A-UAL 187 were determined by Edman degradation. A mixed oligonucleotide probe (24-mer) homologous to the degenerate sequence of the first eight residues at the N-terminus hybridized to a 2.9 kb

*Hpa*II fragment of the 7.6 MDa plasmid from the producer strain, *Leuconostoc gelidum* UAL 187. The fragment was cloned into the *Acc*I site of pUC118 and then subcloned as a *Pst*I-*Sac*I fragment into a lactococcal shuttle vector, pNZ19. DNA sequencing revealed an operon consisting of two open reading frames (ORF) flanked by a putative upstream promoter and downstream terminator. The first ORF downstream of the promoter contains 61 amino acids and was identified as the leucocin structural gene, consisting of a 37 amino acid bacteriocin and a 24 residue N-terminal extension. The second ORF contains 113 amino acids and may produce an immunity protein. Phenotypic expression of the bacteriocin was not achieved in several lactic acid bacteria that were electrotransformed with the hybrid plasmid, pNZ19, containing the 2.9 kb cloned fragment of the leucocin A plasmid.

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"The king's heart is in the hand of the Lord, he directs
it like a watercourse, wherever he pleases." (Solomon)

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1. General Introduction

Nutrient rich foods, such as red meats and poultry, provide excellent environments for microbial growth. Refrigeration is widely used to retard microbial growth in these foods. In chilled meats, psychrotrophic organisms grow and ultimately cause spoilage. In an aerobic environment, nonsporeforming Gram-negative bacteria, including the genera *Pseudomonas*, *Acinetobacter* and (or) *Alcaligenes*, predominate the microflora of meat. Meats have also been implicated in food poisoning outbreaks because of contaminating pathogenic microorganisms including: *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* spp., and *Clostridium botulinum*. More recently, vacuum or modified atmosphere packaging (MAP) has been used to preserve chilled meats, creating an anaerobic environment that favours the growth of lactic acid bacteria (L.A.B.) over the aerobic spoilage microflora or pathogenic bacteria (22, 33).

1.1 Lactic acid bacteria associated with meat

For the purposes of this review, the LAB are considered as those Gram-positive organisms that produce lactic acid as a major end product of metabolism. They include the genera: *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Carnobacterium*, *Leuconostoc*, *Micrococcus*, *Propionibacterium* and *Bifidobacterium*. Among the LAB in vacuum packaged meats, the lactobacilli are dominant (4, 12, 21, 25, 28, 31, 44, 51, 56, 57). The lactobacilli reported in these studies included *Lactobacillus carnis*, *Lactobacillus divergens* and *Lactobacillus piscicola*. These bacteria have subsequently been classified in a new genus, *Carnobacterium* (15). Although the leuconostocs are not reported to be as numerous as lactobacilli in the microflora of MAP meats, many studies have shown that they are prevalent in vacuum packaged meats (31, 56, 59, 64, 70). Leuconostocs are Gram-positive, catalase-negative cocci that produce D(-)-lactic acid as the main end product of metabolism. The main species of leuconostoc found in meat were either *Leuconostoc paramesenteroides* (nondextran-producing and most commonly found in bacon) (12) or

Leuconostoc mesenteroides (dextran forming and most prevalent on vacuum-packaged beef) (31). Recently, two new species of leuconostoc have been described: *Leuconostoc gelidum* and *Leuconostoc carnosum*. These are commonly isolated from chill-stored meats and they are unable to grow at 37°C (65).

Factors contributing to the dominance of lactic acid bacteria in mixed fermentations may be (i) organic acids, (ii) CO_2 (iii) H_2O_2, (iv) diacetyl, and (v) bacteriocins (42). It is the purpose of this review to summarize the information on the bacteriocins produced by the LAB and concentrate on those that are produced by lactic acid bacteria from meat or that have been added to meat to test their preservative effect.

1.2 Definition of bacteriocins

An early definition of bacteriocins produced by Gram-positive bacteria was based on the characteristics of colicins. These characteristics included: (i) a narrow inhibitory spectrum against closely related species, (ii) a biologically active protein moiety, (iii) bactericidal mode of action, (iv) attachment to specific cell receptors, (v) plasmid-borne genetic determinants, and (vi) production by lethal biosynthesis i.e. producing the bacteriocin leads to the death of the producer organism (67). Bacteriocins produced by Gram-positive bacteria that have been characterized often do not fit all of these criteria. For example, Helveticin J produced by *Lactobacillus helveticus* (34) is chromosomally mediated, and Pediocin AcH (10) has a broad spectrum of activity.

A new definition was necessary for Gram-positive bacteriocins. In 1976, Tagg *et al.* (67) stated that they are very difficult to define because many of the inhibitory substances have not been sufficiently characterized to facilitate a good general definition. Renewed interest in bacteriocins of LAB led to the characterization of several of these compounds. In a review published in 1988, Klaenhammer (40) defined bacteriocins as "proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer organism." This is, of necessity, a general definition

because bacteriocins represent a heterogenous group of bacterial antagonists that vary considerably in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties.

1.3 Bacteriocins of LAB

The bacteriocins produced by the LAB were recently reviewed (40). Since this review several new bacteriocins have been identified and studied. The bacteriocins of the LAB are summarized in Table 1.1.

1.4 Bacteriocins produced by meat lactics or with potential for use in meats

1.4.1 Lactococcus spp.

Nisin

Although Nisin A is not produced by a lactic acid bacterium of meat origin, it is worthy of mention because it has been tested in meats as a potential preservative. Nisin A is produced by *Lactococcus lactis* subsp. *lactis* (*L. lactis*) and was the first antimicrobial compound produced by a lactic acid bacterium that showed promise as a food preservative. It has been approved for use in certain foods in over 45 countries, including Britain and the U.S.A. Nisin A is bactericidal to a wide range of Gram-positive bacteria and prevents the outgrowth of *Clostridium* and *Bacillus* spores (32).

Nisin A is a lantibiotic. The lantibiotics are a group of peptide antibiotics that contain lanthionine residues. The knowledge of naturally occurring lantibiotics is increasing and those that have been reported to date are summarized in Table 1.2. Typically, these are polycyclic peptide antibiotics that possess a high content of unsaturated amino acids: dehydroalanine (DHA), dehydrobutyrine (DHB) and thio-ether amino acids [lanthionine (ALA-S-ALA) and ß-methyllanthionine (ABA-S-ALA; ABA = amino butyric acid)].

Table 1.1 A summary of bacteriocins produced by lactic acid bacteria.

Bacteriocin	Producer	Genetic Locus	Mol. Wt. (daltons)	Properties	Ref
a) *Lactobacillus* spp.					
ND[a]	*L. fermenti*	ND	ND	Protein-lipo-carbohydrate	19
Lactocin 27	*L. helveticus* 27	ND	>2,000,000	Protein-lipo-polysaccharide	71
Helveticin J	*L. helveticus*	Chromosome	*ca.* 37,000	333 amino acids	35
Lactacin B	*L. acidophilus*	Chromosome	*ca.* 6,000-6,500		8
Lactacin F	*L. acidophilus*	110 kb plasmid	*ca.* 6,500	57 amino acids	48
Plantaricin A	*L. plantarum*	ND	>8,000		17
Sakacin A	*L. sake*	18 MDa plasmid	ND		61
Lactocin S	*L. sake*	50 kb plasmid	ND	54 amino acids	47
Caseicin	*L. casei*	ND	40,000-42,000		54
b) *Lactococcus* spp.					
Nisin	*L. lactis*	Chromosome/ plasmid	3,354	Lantibiotic 34 amino acids	7
Lacticin 481	*L. lactis*	ND	*ca.* 1,500	Lantibiotic	53
Diplococcin	*L. cremoris*	54 MDa plasmid	*ca.* 5,300		18
Lactostrepcins	*L. lactis*	ND	ND	Acid bacteriocins	41
c) *Carnobacterium* spp.					
Carnobacteriocin	*C. piscicola*	49 MDa plasmid	ND		2
d) *Pediococcus* spp.					
Pediocin AcH	*P. acidilactici*	7.4 MDa plasmid	*ca.* 2,700		55
Pediocin PA-1	*P. acidilactici*	6.2 MDa plasmid	*ca.* 16,500		50
Pediocin A	*P. cerevisiae*	13.6 MDa plasmid	ND		16
e) *Leuconostoc*					
Leucocin A-UAL 187	*L. gelidum*	7.6 MDa plasmid	3,930	37 amino acids	29

[a] ND = not determined

Table 1.2 A summary of the lantibiotics.

Lantibiotic	Molecular Mass	Amino Acids	Microorganism	Ref
Actagardine	1,889	19	*Actinoplanes*	38
Ancovenin	1,959	19	*Streptomyces* sp.	72
Cinnamycin	2,041	19	*Streptomyces cinnamoneus*	9[a]
Duramycin	2,012	19	*Streptomyces cinnamoneus*	66[a]
Epidermin	2,164	22	*Staphylococcus epidermidis*	5
Gallidermin	2,164	22	*Staphylococcus gallinarum*	37
Lanthiopeptin	2,043	19	*Streptoverticullum cinnamoneum*	49
Mersacidin	1,764	19	*Bacillus* sp.	24
Nisin	3,354	34	*Lactococcus lactis*	7
Pep5	3,488	34	*Staphylococcus epidermidis*	58
Ro-09-0198	2,041	19	*Streptoverticullum griseover ticillatum*	39
Subtilin	3,317	32	*Bacillus subtilis*	6

[a] Updating information in reference by Kellner *et al.* (37).

Nisin is the best characterized bacteriocin produced by the lactic acid bacteria. The structural gene of nisin has been cloned and characterized (11, 20, 36). Nisin is synthesized as a pre-propeptide which undergoes post-translational modification to generate the mature peptide. Nucleotide sequences characteristic of an IS element (IS 904) were located upstream of the nisin determinant (20). The nisin gene is just one component of a large transmissible gene block that also encodes sucrose metabolism and nisin resistance. The insertion sequence may play a role in mediating the transfer of this gene block between strains.

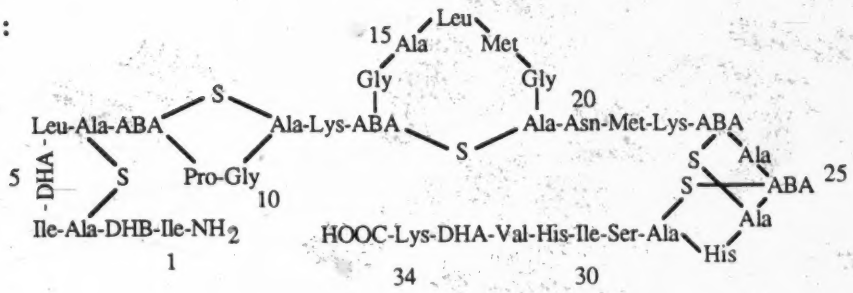
The structural schematic of nisin developed by Gross and Morell (26) is shown in Fig. 1.1 and illustrates that it contains four unusual amino acids: dehydrobutyrine (DHB), dehydroalanine (DHA), lanthionine and ß-methyllanthionine. Chan *et al.* (13) characterized a breakdown product of nisin [(des-ΔAla5)nisin$^{1-32}$] which had lost two carboxy terminal amino acids and the DHA at position 5 that had no biological activity, whereas 100% biological activity was retained by nisin 1-32 which had the DHA at position 5 intact. This indicates that the integrity of the A ring and the DHA at the number 5 position may be involved in biological activity.

It has been suggested that nisin acts by the covalent binding of its dehydroalanine residues to sulphydryl groups on the bacterial cell membrane (45). Henning *et al.* (30) reported a strong interaction of nisin with phospholipids, suggesting the site of attack as the cytoplasmic membrane. The interaction of nisin with phospholipids should be considered before it is used as an additive in food. Emulsifiers, for example, have a high phospholipid content and may inactivate nisin.

Addition of nisin to meats.

The effect of adding pure nisin to meats has been tested in several studies. Its antibotulinal effect was tested in bacon (68) and in a chicken frankfurter emulsion (69). Nisin has proven antibotulinal effectiveness in culture media (63), but the levels of nisin

Nisin:



Propeptide:

I T S I S L C T P G C K T G A L M G C N M K T A T C H C S I H V S K

1 5 10 15 20 25 30 34

Figure 1.1 The amino acid sequences of nisin (26) **and its propeptide** (11).
ABA = amino butyric acid.
DHA = dehydroalanine.
DHB = dehydrobutyrine (ß-methyldehydroalanine).
Ala-S-Ala = lanthionine.
ABA-S-Ala = ß-methyllanthionine.

required to obtain a significant shelf-life extension for bacon were high (100-150 ppm). This could only be attained in conjunction with nitrite (120 ppm). It was concluded that this limited antibotulinal effect in bacon was of little practical value because the reduction in the amount of nitrite was not significant.

The ineffectiveness of nisin was attributed to its poor solubility in brine pickle solutions. The average pH of the bacon samples tested was 6.1, at which nisin is practically insoluble (33). Similar conclusions were drawn when nisin was tested in the chicken frankfurter emulsion. The average pH of the emulsions tested was 6.0 which caused a similar problem with solubility. Nisin was effective on its own at a concentration of 500 ppm but the extension of shelf-life was marginal. The addition of 100 to 250 ppm nisin together with 120 ppm nitrite was superior to addition of 156 ppm nitrite. It was suggested that the antibotulinal effectiveness of nisin-nitrite combinations in cured meats should be further studied and that the relative importance of nisin binding to meats and proteolytic digestion of nisin during processing should be determined.

The effect of adding nisin to raw meat inoculated with several food related bacteria has also been tested (14). Nisin did not inhibit the Gram-negative organisms tested. However, it did delay the growth of *List. monocytogenes* and *Staph. aureus*. The inhibitory effect was greater at refrigeration temperatures. The extractable activity of nisin decreased with increasing temperature. Chung *et al.* (14) concluded that nisin was effective in inhibiting the growth of some Gram-positive organisms in meat. However, its overall value in preventing meat spoilage was limited because of its inability to inhibit the Gram-negative spoilage flora.

From these studies it appears that nisin has a modest antibotulinal effect in meat. Also, unless the problems of insolubility at the pH of cured meats is solved, nisin will have little potential for use. The high phospholipid content in emulsifiers may also be a factor in reducing the effectiveness of nisin. As yet, there are no reports of the inoculation of a nisin-producing organism into a meat system to produce nisin in situ.

1.4.2 Lactobacillus spp.

Schillinger and Lücke (61) reported that 10% of strains of *Lactobacillus* isolated from meat and meat products showed antagonistic activity. Most of these strains were *Lactobacillus sake* and one of them (*Lb. sake* Lb706) was further characterized. The inhibitory substance was shown to be proteinaceous and there was physical evidence to suggest that bacteriocin production and immunity was associated with an 18 MDa plasmid. This bacteriocin was designated Sakacin A, but it has not been further characterized. It is active against other lactic acid bacteria as well as *List. monocytogenes*.

Initial in vitro competitive studies indicated that an organism producing this bacteriocin could effectively inhibit a bacteriocin-sensitive strain when they grow together. In a subsequent study (43), *Lb. sake* Lb706 and a bacteriocin-negative mutant, *Lb. sake* Lb706-B, were added to samples of pasteurized minced meat that was inoculated with a sensitive strain of *List. monocytogenes*. In the meat sample containing the bacteriocin producer, the *Listeria* count was reduced by *ca.* one log cycle within 2 days. This was not observed in the sample containing the bacteriocin-negative mutant. The presence of either strain of *Lb. sake* prevented the growth of *Listeria* for 6 days at 8°C, whilst the control sample showed an increase in numbers of *Listeria* of 3 log cycles over this time period. Problems associated with the use of sakacin A were: the inhibitory effect in meat was much less than that observed in broth; sakacin A was slowly inactivated over time; and the spectrum of activity does not include all of the Gram-positive bacteria of concern in meats.

Lactocin S is a bacteriocin produced by *Lb. sake* strain L45 isolated from naturally fermented dry sausage (46). This bacteriocin is active against strains of closely related genera of *Lactobacillus*, *Leuconostoc* and *Pediococcus*. It has been purified and the amino acid sequence of 54 residues is known. A 50 kb plasmid is associated with bacteriocin production and immunity. Plasmid rearrangements during conjugation experiments suggest the involvement of a transposon. There are no reports concerning the ability of this bacteriocin to preserve meats.

1.4.3 Carnobacterium spp.

Bacteriocin production by carnobacteria has been reported on several occasions (1, 60, 62). This is a newly described genus that is differentiated from the genus *Lactobacillus* on the basis that these bacteria are unable to grow on acetate agar (pH 5.6), grow at high pH (8.5-9.5) and producte L(+) lactate. In 1988, Schoebitz (62) inoculated *Carn. piscicola* onto the surface of meat and detected inhibitory activity on the surface of the meat. Ahn and Stiles studied bacteriocin production by *Carn. piscicola* LV17 (1, 2). At least two bacteriocins are produced by this organism and both are detected early in the growth phase of the bacterium. The bacteriocins are not produced in media with pH adjusted below 5.5. The bacteriocins are inactivated by several proteolytic enzymes and are stable over a wide range of pH and temperature conditions, including heating at 100°C for 30 min. These bacteriocins have a bactericidal mode of action against a *Carnobacterium* indicator strain (*Carn. divergens* LV13) and need contact between the cytoplasmic membrane and cell wall for the lethal effect to occur.

Initial genetic studies revealed that bacteriocin production was mediated by two plasmids (3). Bacteriocin production encoded by a 49 MDa plasmid was responsible for 80% of the total bacteriocin activity whilst the remaining 20% was encoded by a 40 MDa plasmid. The bacteriocins have different specificities and are active mainly against other lactic acid bacteria, as well as strains of *List. monocytogenes* and *Ent. faecalis*. Studies using a comobilization conjugation technique to transfer and isolate plasmids confirmed the association of the 40 and 49 MDa plasmids with bacteriocin production and immunity. A cointegrate of the 40 and 49 MDa plasmids was also found after conjugal transfer. Full bacteriocin production and immunity was acquired with this new 89 MDa co-integrate. After attempted transfer of the 89 MDa plasmid, two new plasmids were evident in a transconjugant showing full bacteriocinogenicity. A 9.6 kb region of the 49 MDa plasmid was cloned and shown to be responsible for bacteriocin production and immunity. Competitive studies using this bacteriocin in broth and meat systems are in progress.

Schillinger and Holzapfel studied 38 strains of carnobacteria of which 18 strains showed inhibitory activity against other *Carnobacterium* spp. and *Lactobacillus* spp. The inhibitory activity of *Carn. divergens* L66 was studied in greater detail. The inhibitory substance produced by this strain was inactivated by trypsin but not by heat treatment at 100°C for 10 min. A tenfold concentrate was active against several strains of *Ent. faecalis* and *List. monocytogenes*. The competitive ability of *Carn. divergens* L66 in broth or in meat was not studied.

1.4.4 Pediococcus spp.

Several pediococci have been shown to produce bacteriocins, including *Pediococcus cerevisiae* FBB-61 and L-7230 (23) and *Pediococcus Pentosaceus* FBB-61 and L-7230 of vegetable origin (16). Further studies on *Ped. pentosaceus* FBB-61 (17) showed that it produced an inhibitory substance that was plasmid-mediated and that it was active against several important foodborne pathogens, including *Cl. botulinum*, *Staph. aureus* and *List. monocytogenes* (40).

The only bacteriocin produced by a *Pediococcus* of meat origin that has been studied is pediocin AcH produced by *Ped. acidilactici* H. The organism was isolated from fermented sausage (10) and exhibited inhibitory activity against *Staph. aureus*, *Brochothrix thermosphacta*, *Clostridium perfringens* and *List. monocytogenes*. It was also active against two species of Gram-negative bacteria, *Pseudomonas putida* and *Aeromonas hydrophila*. Pediocin AcH was purified and appeared as a band running at *ca.* 2,700 daltons on SDS-PAGE. It was associated with a 7.4 MDa plasmid by conjugation experiments (55). The crude bacteriocin was active over a wide pH range and resistant to heating at 93°C for 15 min. at pH levels ranging from 2.0 - 9.0. It has a bactericidal mode of action but it does not induce lysis or increased cell wall permeability. There have been no studies reported in which this organism or bacteriocin has been added to meat to determine its competitive effectiveness.

However, Pediocin PA-1 produced by *Ped. acidilactici* PAC 1.0 has been tested in meats. Production of this bacteriocin is associated with a 6.2 MDa plasmid. The bacteriocin has a molecular weight of *ca.* 16,500 determined by gel filtration. It has a bactericidal mode of action but its spectrum of activity is limited to closely related organisms amongst the pediococci, lactobacilli and leuconostocs. Neilsen *et al.* (50) added a crude solution of Pediocin PA-1 to meat that had been inoculated with *List. monocytogenes*. The bacteriocin reduced the number of attached bacteria by 0.5 to 2.2 log cycles depending upon the concentration of pediocin PA-1. When meat was treated with pediocin PA-1 prior to inoculation with *List. monocytogenes*, 1.0 to 2.5 log fewer bacteria were detected than were detected in the control. A suspected problem of adding bactericidal peptides to a meat system is their inactivation by proteases inherent in the meat. However, this study indicated that there was no breakdown of the pediocin PA-1 in the meat.

1.4.5 Leuconostoc spp.

Production of bacteriocin-like substances by the leuconostocs has been reported (27, 52) but nothing is known about the physiochemical nature of these substances. Harding and Shaw (27) reported that *Leuc. gelidum* (65) produced an inhibitory substance that was active against lactobacilli, leuconostocs and three strains of *List.monocytogenes*, but it did not inhibit a range of Gram-negative or sporeforming Gram-positive bacteria that were tested. The antagonistic activity was inactivated by protease and survived heat treatment of 100°C for 60 min. A molecular weight of greater than 10,000 was suggested on the basis of retention during dialysis.

In conclusion, renewed interest in the antimicrobial activities of the LAB has resulted in the study of bacteriocins produced by a wide variety of these organisms. The bacteriocins from LAB of dairy origin have been more extensively investigated than those of meat origin. However, apart from nisin, the precise characterization of these anti-

microbial peptides is in its infancy and further study is required to elucidate the mechanisms of production and activity of these compounds.

The objective of the study described in this thesis was to characterize an inhibitory substance produced by a strain of *Leuconostic gelidum* UAL 187. This study is divided into 3 main parts:

1) Initial studies to characterize the producer organism, to identify physiological characteristics of production of the bacteriocin and to determine whether the genetic location of the bacteriocin was on one of the 3 plasmids harboured by the organism (Chapter 2).
2) Isolation, purification and characterization of the bacteriocin, leucocin A-UAL 187 (Chapter 3).
3) Identification, cloning and analysis of the genetic determinants of the bacteriocin (Chapter 4).

The characterization of the bacteriocin was done in order to elucidate the structure and genetic control of this bacteriocin at the molecular level, to provide a basis for understanding the relationship between structure and function of these compounds.

1.5 References

1. Ahn, C., and M.E. Stiles. 1990. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. J. Appl. Bacteriol. 69:302-310.
2. Ahn, C., and M.E. Stiles. 1990. Plasmid associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Appl. Environ. Microbiol. 56:2503-2510.
3. Ahn, C., and M.E. Stiles. 1991. Mobilization of plasmids associated with bacteriocin production in *Carnobacterium piscicola* by pAMß1. Appl. Environ. Microbiol. In Press.
4. Allen, J.R., and E.M. Foster. 1960. Spoilage of vacuum-packaged sliced processed meat during refrigerated storage. Food Research 25:19-25.
5. Allgaier, H., G. Jung, R.G. Werner, U. Schneider, and H. Zahner. 1986. Epidermin: sequencing of a heterodetic tetracyclic 21-peptide amide antibiotic. Eur. J. Biochem. 160:9-22.
6. Bannerjee, S., and N.J. Hansen. 1988. Structure and expression of a gene encoding the precursor of subtilin, a small peptide antibiotic. J. Biol. Chem. 263:9508-9514.
7. Barber, M., G.J. Elliot, R.S. Bordoli, B.N. Green, and B.W. Bycroft. 1987. Confirmation of the structure of nisin and its major degradation product by FAB-MS and FAB MS/MS. Experientia 44:266-270.
8. Barefoot, S.F., and T.R. Klaenhammer. 1984. Purification and characterization of the *Lactobacillus acidophilus* bacteriocin Lactacin B. Antimicrob. Agents Chemother. 26:328-334.
9. Benedict, R.G., W. Dwonch, O.L. Shotwell, T.G. Pridham, and L.A. Lindenfelser. 1952. Cinnamycin, an antibiotic from *Streptomyces cinnamoneus* nov. sp. Antibiotic. Chemother. 2:591-594.

10. Bhunia, A.K., M.C. Johnson, and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. J. Appl. Bacteriol. 65:261-268.

11. Buchman, W.B., S. Banerjee, and J.N. Hansen. 1988. Structure, expression and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. J. Biol. Chem. 263:16260-16266.

12. Cavett, J.J. 1963. A diagnostic key for identifying the lactic acid bacteria of vacuum-packed bacon. J. Appl. Bacteriol. 26:453-470.

13. Chan, W.C., B.W. Bycroft, L.Y. Lian, and G.K. Roberts. 1989. Isolation and characterization of two degradation products derived from the peptide antibiotic nisin. FEBS. 252:29-36.

14. Chung, K.T., J.S. Dickson, and J.D. Crouse. 1989. Effects of nisin on growth of bacteria attached to meat. Appl. Environ. Microbiol. 55:1329-1333.

15. Collins, M.D., J.A.E. Farrow, B.A. Phillips, S. Ferusu, and D. Jones. 1987. Classification of *Lactobacillus divergens*, *Lactobacillus piscicola* and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus *Carnobacterium*. Int. J. Syst. Bacteriol. 37:310-316.

16. Daeschel, M.A., and T.R. Klaenhammer. 1985. Association of a 13.6 megadalton plasmid in *Pediococcus pentosaceus* with bacteriocin activity. Appl. Environ. Microbiol. 50:1538-1541.

17. Daeschel, M.A., M.C. McKenny, and L.C. McDonald. 1986. Characterization of a bacteriocin from *Lactobacillus plantarum*. Abstracts of the Annual Meeting of the American Society for Microbiology. 86:277.

18. Davey, G.P. 1981. Mode of action of diplococcin, a bacteriocin from *Streptococcus cremoris* 346. New Zealand J. Dairy Sci. Technol. 16:187-190.

19. DeKlerk, H.C., and J.A. Smit. 1967. Properties of *Lactobacillus fermenti* bacteriocin. J. Gen. Microbiol. 48:309-316.

20. Dodd, H.M., N. Horn, and M.J. Gasson. 1990. Analysis of the genetic determinant for production of the peptide antibiotic nisin. J. Gen. Microbiol. 136:555-566.

21. Egan, A.F. 1983. Lactic acid bacteria of meat and meat products. Antonie van Leeuwenhoek 49:327-336.

22. Farber, J.M., D.W. Warburton, L. Gour, and M. Milling. 1990. Microbiological quality of foods packaged under modified atmospheres. Food Microbiol. 7:327-334.

23. Fleming, H. P., J.L.Etchells, and R.N. Costilow, R.N. 1975. Microbial inhibition by an isolate of pediococcus from cucumber brine. Appl. Environ. Microbiol. 3:1040-1042.

24. Ganguli, B.N., S. Chatterjee, S. Chatterjee, H. Kogler, H.W. Fehlhaber, N. Klesel, and J. Blumbach. 1989. Mersacidin. A novel peptide antibiotic. Discovery and microbial evaluation. Program and Abstracts of the 29th Intersci. Conf. on Anti-microb. Agents and Chemother., No. 413, p. 169.

25. Gardner, G.A. 1968. Effects of pasteurization or added sulphate on the microbiology of stored vacuum-packed bacon burgers. J. Appl. Bacteriol. 31:462-478.

26. Gross, E., and J.L. Morell. 1971. The structure of nisin. J. Am. Chem Soc. 93:4634-4635.

27. Harding, C.D., and B.G. Shaw. 1990. Antimicrobial activity of *Leuconostoc gelidum* against closely related species and *Listeria monocytogenes*. J. Appl. Bacteriol. 69:648-654.

28. Hastings, J.W., and W.H. Holzapfel. 1987. Conventional taxonomy of lactobacilli surviving the radurization of meat. J. Appl. Bacteriol. 62:209-216.

29. Hastings, J.W., and M.E. Stiles. 1991. Antibiosis of *Leuconostoc gelidum* isolated from meat. J. Appl. Bacteriol. 70:127-134.

30. Henning, S., R. Metz, and W.R. Hammes. 1986. Studies on the mode of action of nisin. Int. J. Food Microbiol. 3:121-134.

31. Hitchener, B.J., A.F. Egan, and P.J. Rogers. 1982. Characteristics of lactic acid bacteria isolated from vacuum-packaged beef. J. Appl. Bacteriol. 52:31-37.

32. Hurst, A. 1981. Nisin. Adv. Appl. Microbiol. 27:85-123.

33. ICMSF. 1980. Microbial Ecology of Foods. Vol. 2 (Silliker, J.W., ed.). Academic Press, New York.

34. Joerger, M.C., and T.R. Klaenhammer. 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Bacteriol. 167:439-446.

35. Joerger, M.C., and T.R. Klaenhammer, 1990. Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. J. Bacteriol. 172:6339-6347.

36. Kaletta, C., and K.D. Entian. 1989. Nisin, a peptide antibiotic: Cloning and sequencing of the nisA gene and post-translational processing of its peptide product. J. Bacteriol. 171:1594-1601.

37. Kellner, R., G. Jung, T. Horner, H. Zahner, N. Schnell, K.D. Entian, and F. Gotz. 1988. Gallidermin: a new lanthionine - containing polypeptide antibiotic. Eur. J. Biochem. 177:53-59.

38. Kettenring, J.K., A. Malabaiba, K. Vekey, and B. Cavalleri. 1990. Sequence determination of actagardine, a novel lantibiotic, by homonuclear 2D NMR spectroscopy. J. Antibiotics 43:1082-1088.

39. Kessler, H., S. Steuernagel, D. Gillesen, and T. Kamiyama. 1987. Complete sequence determination and localisation of one imino and three sulfide bridges of the nonadecapeptide Ro 09-0198 by homonuclear 2D-NMR spectroscopy. The DQF-RELAYED-NOESY-Experiment. Helvetica Chimica Acta. 70:726-741.

40. Klaenhammer, T.R. 1988. Bacteriocins of lactic acid bacteria. Biochimie 70:337-349.

41. Kozak, W., J. Bardowski, and W.T. Dobrzanski. 1978. Lactostrepcins - acid bacteriocins produced by lactic streptococci. J. Dairy Res. 45:247-257.

42. Lindgren, S.E., and W.J. Dobrogosz. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 87:149-164.

43. Lücke, F.K., and U. Schillinger. 1990. Possible use of bacteriocin-producing lactobacilli in meats. FEMS Microbiol. Rev. 87:abstr. E3.

44. Mol, J.H.H., J.A.E. Hietbring, H.W.M. Mollen, and J. Van Tinterin. 1971. Observations on the macroflora of vacuum-packed sliced cooked meat products. J. Appl. Bacteriol. 34:377-397.

45. Morris, S.L., R.C. Walsh, and J.N. Hansen. 1984. Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. J. Biol. Chem. 259:13590-13594.

46. Mortvedt, C.I., and I.F. Nes. 1990. Plasmid-associated bacteriocin production by a *Lactobacillus sake* strain. J. Gen. Microbiol. 136:1601-1607.

47. Mortvedt, C.I., J. Nissen-Meyer, and I.F. Nes. 1990. Purification of a bacteriocin from *Lactobacillus sake* and its association with a 50 kb plasmid. FEMS Microbiol. Rev. 87: abstr. E17.

48. Muriana, P.M., and T.R. Klaenhammer. 1991. Purification and partial characterization of Lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. Appl. Environ. Microbiol. 57:114-121.

49. Naruse, N., O. Tenmyo, K. Tomita, M. Konishi, T. Miyaki, and H. Kawaguchi. 1989. Lanthiopeptin, a new peptide antibiotic. Production, isolation and properties. J. Antibiotics. 42:837-845.

50. Neilsen, J.W., J.S. Dickson, and J.D. Crouse. 1990. Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. Appl. Environ. Microbiol. 56:2142-2145.

51. Niemand, J.G., and W.H. Holzapfel. 1984. Characteristics of lactobacilli isolated from radurized meat. Int. J. Food Microbiol. 1:99-110.

52. Orberg, P.K., and W.E. Sandine. 1984. Common occurrence of plasmid DNA and vancomycin resistance in *Leuconostoc* spp. Appl. Environ. Microbiol. 48:1129-1133.

53. Piard, J.C., P.M. Muriana, M. Desmazeaud, and T.R. Klaenhammer. 1990. Purification of Lacticin 481, a bacteriocin produced by *Lactococcus lactis* CNRZ 481. FEMS. Microbiol. Rev. 87: Abstr. E14.

54. Rammelsberg, M., E. Muller, and F. Radler. 1990. Caseicin 80: Purification and characterization of a new bacteriocin from *Lactobacillus casei*. Arch. Microbiol. 154:249-252.

55. Ray, S.K., M.C. Johnson, and B. Ray. 1989. Bacteriocin plasmids of *Pediococcus acidilactici*. J. Indust. Microbiol. 4:163-171.

56. Reuter, G. 1970. Laktobazillen und eng verwandte Mikroorganismen in Fleisch und Fleischwaien. 4 Mitteilung: Die Okologie von Laktobazillen, Leuconostoc-species und Pediokokken. Fleischwirtschaft. 50:1397-1399.

57. Reuter, G. 1975. Classification problems, ecology and some biochemical activities of lactobacilli of meat products. In: Lactic Acid Bacteria in Beverages and Food. ed. Can, J.C., G.V. Cutting and G.C. Whiting. pp. 221-229. London. Academic Press.

58. Sahl, H.G., and H. Brandis. 1981. Production, purification and chemical properties of an antistaphylococcal agent produced by *Staphylococcus epidermidis*. J. Gen. Microbiol. 127:377-384.

59. Savell, J.W., M.O. Hanna, C. Vanderzant, and G.C. Smith. 1981. An incident of predominance of *Leuconostoc* sp. in vacuum-packaged beef strip loins - sensory and microbial profiles of steaks stored in O_2-, CO_2- and N_2- atmospheres. J. Food Prot. 44:742-745.

60. Schillinger, U., and W.H. Holzapfel. 1990. Antibacterial activity of carnobacteria. Food Microbiol. 7:305-310.

61. Schillinger, U., and F.K. Lücke. 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. 55:1901-1906.

62. Schoebitz, R.P. 1988. Production of inhibitory substances by lactic acid bacteria in ground beef. M.Sc. Thesis. University of Alberta, Edmonton, Alberta, Canada.

63. Scott, V.N., and S.L. Taylor. 1981. Effect of nisin in the outgrowth of *Clostridium botulinum* spores. J. Food Sci. 46:117-120, 126.

64. Shaw, B.G., and C.D. Harding. 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. J. Appl. Bacteriol. 56:25-40.

65. Shaw, B.G., and C.D. Harding. 1989. *Leuconostoc gelidum* sp. nov. and *Leuconostoc carnosum* sp. nov. from chill-stored meats. Int. J. Syst. Bacteriol. 39:217-223.

66. Shotwell, O.L., F.H. Stodola, W.R. Michael, L.A. Lindenfelser, R.G. Dworschack, and T.G. Pridham. 1958. Antibiotics against plant disease. III. Duramycin, a new antibiotic from *Streptomyces cinnamoneus* forma azacoluta. J. Am. Chem. Soc. 80:3912-3915.

67. Tagg, J.R., A.S. Dajani, and L.W. Wannamaker. 1976. Bacteriocins of Gram-positive bacteria. Bacteriol. Rev. 40:722-756.

68. Taylor, S.L., and E.B. Somers. 1985. Evaluation of the antibotulinal effectiveness of nisin in bacon. J. Food Prot. 48:949-952.

69. Taylor, S.L., E.B. Somers, and L.A. Krueger. 1984. Antibotulinal effectiveness of nisin-nitrate combinations in culture medium and chicken frankfurter emulsions. J. Food Prot. 48:234-239.

70. Tonge, R.J., A.C. Baird-Parker, and J.J. Cavett. 1964. Chemical and microbiological changes during storage of vacuum-packed bacon. J. Appl. Bacteriol. 27:252-264.

71. Upreti, G.C., and R.D. Hinsdill. 1975. Production and mode of action of lactocin 27: Bacteriocin from a heterofermentative *Lactobacillus*. Antimicrob. Agents Chemother. 7:139-145.

72. Wakamiya, T., Y. Ueki, T. Shiba, Y. Kido, and Y. Motoki. 1985. The structure of ancovenin, a new peptide inhibitor of angiotensin I converting enzyme. Tetrahedron Lett. 26:665-668.

2. Antibacterial Activity Associated with *Leuconostoc gelidum* Isolated from Meat[1]

2.1 Introduction

Refrigerated meats stored under vacuum or in modified atmospheres containing elevated levels of carbon dioxide develop a microbial population that consists predominantly of lactic acid bacteria, in contrast to the Gram-negative spoilage bacteria that develop under aerobic conditions of refrigerated storage. Shaw and Harding (22) identified three main clusters of lactic acid bacteria among 100 strains isolated from vacuum-packaged meats. These included aciduric and nonaciduric *Lactobacillus*-type organisms and leuconostocs. Shaw and Harding (23) also described two new *Leuconostoc* species from chill-stored meats: *Leuconostoc gelidum* and *Leuconostoc carnosum*, both of which are unable to grow at 37°C.

The antimicrobial properties of lactic acid bacteria are of special interest to researchers involved in developing strongly competitive starter cultures for food fermentations. In a mixed fermentation environment, production of bacteriocins may provide the competitive advantage for a producer organism to dominate the microbial population. Bacteriocins, by definition, are antibacterial proteins or protein complexes that are generally active against closely related species (24). Bacteriocin production is a widespread phenomenon among lactic acid bacteria (14). It has been observed among the lactobacilli (6), lactococci (10) and pediococci (5). Production of bacteriocin-like compounds by *Leuconostoc* spp. of dairy and wine origin was previously described (19); and, the inhibitory action of *Leuconostoc cremoris* by nonproteinaceous substances, such as organic acids, is well known (3). Schillinger and Lücke (21) reported the production of bacteriocin by *Lactobacillus sake* isolated from meat, but there is no record of bacteriocin production by *Leuconostoc* spp. of meat origin.

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In addition, production and resistance phenotypes of bacteriocins have potential for use as markers in genetic linkage and transfer studies in a group of bacteria where relatively little is known about these systems (14). Production of bacteriocins in some strains of lactic acid bacteria has been associated with chromosomal determinants (4, 13). In other strains, however, loss of a plasmid has been associated with loss of bacteriocin activity (11, 17, 21). In the present study, a lactic acid bacterium showing inhibitory activity against other lactic acid bacteria was isolated from meat packaged under modified atmosphere with elevated CO_2. Characteristics of the inhibitory substance, optimal conditions for its production and the probable genetic determinants of the substance were studied.

2.2 Materials and methods

2.2.1 Bacterial cultures and media

The *Leuconostoc* strain UAL187, that produces an inhibitory substance, was isolated from lactobacilli MRS agar plates used to enumerate the lactic acid bacteria growing on processed meat packaged under elevated CO_2 (30%) and stored at 4°C for six weeks. The bacterium was classified according to criteria described by Schillinger and Lücke (20) and Shaw and Harding (23). The bacterial indicator strain (other than those used in the antagonistic spectrum studies) was *Carnobacterium divergens* strain LV13 supplied by Dr. B.G. Shaw (Institute of Food Research, Langford, Bristol, U.K.). These bacteria were maintained in Cooked Meat Medium (Difco) at 4°C. Test organisms were subcultured at least twice, but not more than five times, in APT broth (Difco) before use in experiments. APT broth was used as the growth medium in experiments and incubation was at 25°C, unless otherwise stated.

2.2.2 Taxonomic classification

Fermentation of carbohydrates was determined with a 1% inoculum of bacterial culture in 5 ml of lactobacilli MRS broth, prepared according to DeMan *et al.* (7) without

glucose or sodium citrate, containing 0.5% carbohydrate and 0.004% chlorophenol red. All tubes were checked for acid production after incubation at 25°C for 24, 48 and 120 h. The carbohydrates included: arabinose, cellobiose, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose and xylose. Cultures were tested for catalase production, hydrolysis of arginine, gas (CO_2) production from glucose and slime (dextran) production from sucrose (20). The configuration of the lactic acid enantiomer was determined enzymatically according to the methods described by Gawehn (8) and Noll (18) using D(-)- and L(+)-lactate-dehydrogenase (Boehringer Mannheim, Dorval, Quebec, Canada). A 24 h culture of the test organism UAL187 grown in APT broth was used as the sample.

2.2.3 Assays for inhibitory substance

(1) Deferred antagonism assay

The method described by Barefoot and Klaenhammer (4) was used, except that APT agar plates were used and they were inoculated using a Cathra replicating inoculator (KVL Laboratories, Cambridge, Ontario, Canada).

(2) Spot-on-lawn assay

A culture of bacteria was adjusted to pH 6.5 with 10 N NaOH and centrifuged at 6,000 x g for 5 min. Microbial cells were inactivated by mixing 1 part chloroform to 4 parts supernatant fluid. After standing for 5 min, the aqueous phase was removed aseptically and 20 µl was spotted on the surface of an APT agar plate overlayered with 6 ml of APT soft agar (0.75%) inoculated with 1% of an overnight culture of indicator organism. Controls with sterile APT broth were used to be certain that no chloroform residues were present.

(3) Arbitrary activity unit assay

The reciprocal of the highest dilution (in a series of doubling dilutions) showing a zone of inhibition by spot-on-lawn technique was taken as the number of arbitrary units of inhibitor, and adjusted to obtain units per ml (AU/ml).

2.2.4 Concentration of inhibitory substance

Ammonium sulphate (390 g/l, 60% saturation) was dissolved in the supernatant fluid of 1 l of the producer strain UAL187 grown in APT broth at 25°C for 48 h. The solution was stirred gently for 24 h and then centrifuged at 6,000 x g for 10 min. The pellet was dissolved in 20 ml of 50 mM phosphate buffer (pH 6.5) and dialyzed in Spectrum no. 1 tubing (molecular weight cut off 6,000 to 8,000; Fisher Scientific Co.) against a 1 l volume of the same buffer at 2°C overnight with two changes of the buffer solution.

2.2.5 Effect of pH and temperature on production of inhibitory substance

Tubes of APT broth adjusted to pH levels between 4.0 and 6.5 at 0.5 intervals were prepared with 10 M HCl. Individual sets of pH-adjusted broth were tempered at 1, 5 and 25°C for 1 h before adding 1% of an overnight culture of the test organism UAL187. Samples were removed at specified time intervals and analyzed for bacterial growth (O.D. 620 nm) and inhibitory activity.

2.2.6 Preliminary characterization of inhibitory substance

Supernatant fluid of the producer strain UAL187, grown overnight in APT broth, was prepared in the same way as described for the spot-on-lawn assay and divided into 1 ml amounts and either untreated (control) or treated with pronase E (Sigma, 1 mg/ml), trypsin (Sigma, 1 mg/ml), catalase (Fisher Scientific, 100 units/ml) or heat (62°C for 30 min). The spot-on-lawn assay was done to determine the presence or absence of the inhibitor.

2.2.7 Killing kinetics

A 0.1 ml sample of an overnight culture of *Carn. divergens* LV13 was added to 100 ml of APT broth and 100 ml of phosphate buffer (50 mM, pH 6.5). These samples were mixed and divided into 50 ml volumes to which 0.1 ml of concentrated inhibitory substance was added to give a final concentration of 100 AU/ml. Samples with no inhibitor added were used as controls. Duplicate samples were plated on APT agar at specified time intervals to determine the number of survivors of indicator strain LV13. After plating on APT agar, the dilutions were treated with protease to give a final concentration of 1 mg/ml and again plated onto APT agar.

2.2.8 Spectrum of antibacterial activity

A spot-on-lawn activity assay using the concentrated inhibitory substance (6,400 AU/ml) from the producer strain UAL187 was done on the following range of indicator strains obtained from the American Type Culture Collection (ATCC), Dr. B.G. Shaw, (LV or SML) and our own meat strains (UAL): *Leuconostoc mesenteroides* ATCC 23368, SML9 and 52, and UAL100, 182 and 187; *Lactobacillus plantarum* ATCC 4008; *Lactobacillus viridescens* ATCC 12706; aciduric *Lactobacillus* strains UAL3 and 4, LV36 and 69; *Pediococcus acidilactici* ATCC 8042; *Ped. parvulus* ATCC 1937; *Carnobacterium piscicola* strains LV17, LV61 and UAL26; *Carn. divergens* LV13; *Enterococcus faecalis* ATCC 19433; *Staphylococcus aureus* ATCC 25923; *Salmonella typhimurium* ATCC 13311; *Pseudomonas fluorescens* ATCC 13525; *Brochothrix thermosphacta* ATCC 11059; and *Listeria monocytogenes* ATCC 15313.

2.2.9 Plasmid detection

A modification of the miniscale plasmid isolation procedure of Anderson and McKay (2) was used. After addition of 5 ml of NaCl, the sample was treated with pronase E (40 µl of 20 mg/ml, Sigma) before phenol extraction. Gel electrophoresis was done as described by Maniatis *et al.* (16). Covalently closed circular DNA was differentiated from

open circular DNA by the method described by Hintermann *et al.* (12). The plasmids of *Escherichia coli* V517 were used as molecular weight standards as described by Macrina *et al.* (15).

2.2.10 Plasmid curing and mutant screening

Novobiocin (Sigma) was diluted in APT broth containing 0.002% sodium dodecyl sulphate (SDS) to give final concentrations of 0 to 25 µg/ml at 5 µg/ml intervals, inoculated with approximately 1 x 10^4 cells of *Leuc. gelidum* UAL187 per ml and incubated at 25°C. When there was visible growth, appropriate dilutions of this sample were spread on APT agar plates and incubated for 24 h. As soon as colonies were visible, plates containing less than 30 colonies were overlayered with 10 ml of soft APT agar containing 1% of a 24 h culture of indicator strain LV13. Colonies that did not produce a zone of inhibition and those with a much reduced zone were plated on sucrose agar plates to detect dextran formation and thus to differentiate between indicator strain LV13 and nonbacteriocinogenic UAL187 derivatives. Dextran forming colonies were isolated and their plasmid profiles were determined. Strains showing loss of one or more plasmids were confirmed by three replicates of plasmid isolations and by Gram stain, catalase test, arginine hydrolysis, gas production from glucose, fermentation of carbohydrates (arabinose, fructose, glycerol, mannitol, ribose and trehalose) and dextran production using methods described under taxonomic classification. Supernatant fluids of cultures of UAL187 variants grown in APT broth were checked for inhibitory activity by both the spot-on-lawn and deferred antagonism techniques against indicator strain LV13 and for immunity to the inhibitory substance.

2.3 Results

The Gram-positive, coccoid-shaped, catalase-negative strain UAL187 was classified as *Leuc. gelidum* based on the following phenotype: production of gas from glucose; inability to hydrolyse arginine; predominance of D(-)-lactate (>90%) and the follow-

ing carbohydrate fermentation pattern [positive (+), negative (-), delayed (d)]: arabinose (+), cellobiose (+), fructose (-), galactose (-), glucose (+), glycerol (-), inositol (-), lactose (d), maltose (+), mannitol (-), mannose (+), melezitose (-), melibiose (+), raffinose (+), rhamnose (-), ribose (+), salicin (-), sorbitol (-), sucrose (+), trehalose (+) and xylose (+). The organism grows over the temperature range of 0 to 30°C, but not at 35°C.

Leuc. gelidum UAL187 showed inhibitory activity against a wide variety of lactic acid bacteria (Table 2.1). It was active against all leuconostocs, lactobacilli, pediococci and carnobacteria tested and a strain of *Ent. faecalis*. No inhibitory zones were evident on lawns of *Staph. aureus*, *Salm. typhimurium*, *Ps. fluorescens* and *Broch. thermosphacta* when spotted with 20 ml of pH-adjusted (pH 6.5) and chloroform-treated supernatant fluid from a 24 h APT broth culture of *Leuc. gelidum* UAL187. An inhibitory zone was observed on a lawn of *Listeria monocytogenes* ATCC 15313 cells when treated in the same manner.

The inhibitory substance produced by *Leuc. gelidum* UAL187 was partially purified with ammonium sulphate to give an 8-fold concentration to 51,000 AU/ml after dialysis. As illustrated in Figure 2.1, the inhibitory substance was sensitive to treatment with protease and trypsin, but not catalase. Activity was not lost when the sample was heated at 62°C for 30 min. Production of inhibitory substance is detected early in the growth cycle, within 3 h of incubation of a 1% inoculum in APT broth incubated at 25°C.

Figure 2.2 shows the growth and production of the inhibitory substance by *Leuc. gelidum* UAL187 in APT broth adjusted to pH values between 4.0 and 6.5, and incubated at 25°C. In all cases, except for APT adjusted to pH 4.0, inhibitory action could be detected in the supernatant fluid after incubation for 3 h. Growth at pH 4 was marginal, but after incubation for 6 h it could be detected. The maximum concentration of inhibitory substance (6,400 AU/ml) was reached after incubation for 48 h in APT broth at pH 6.0 and 6.5 Thereafter, the amount of inhibitory substance in the supernatant fluid declined steadily at

Table 2.1. Relative sensitivity of a variety of indicator organisms to concentrated inhibitor (6,400 AU/ml measured against *Carnobacterium divergens* LV13) produced by *Leuconostoc gelidum* strain UAL187 and adjusted to pH 6.5.

Indicator strain		Highest inhibitory dilution[a]
Leuconostoc spp.		
L. mesenteroides	ATCC 23368	128
	SML9	32
	SML52	64
L. gelidum UAL187		None
Leuconostoc sp. UAL100		4
Lactobacillus spp.		
L. plantarum ATCC 4008		32
L. viridescens ATCC 12706		0
Aciduric *Lactobacillus* sp.	UAL3	16
	UAL4	16
	LV36	32
	LV69	16
Pediococcus spp.		
P. acidilactici ATCC 8042		16
P. parvulus ATCC 1937		128
Carnobacterium spp.		
C. piscicola	LV17	32
	LV61	64
	UAL26	128
C. divergens	LV13	128
Enterococcus sp.		
E. faecalis ATCC 19433		128
Listeria sp.		
L. monocytogenes ATCC 15313		512

[a] Determined by spot-on-lawn technique with an overlay of each indicator organism.

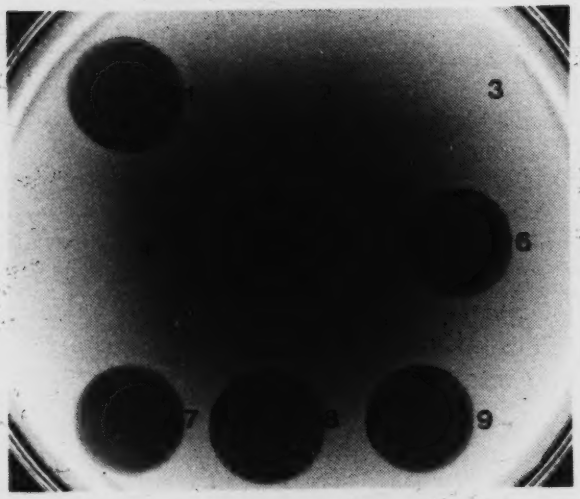

Figure 2.1. Inhibition zones produced by sterilized and pH adjusted (pH 6.5) supernatant fluids of *Leuconostoc gelidum* UAL187 and variants on a lawn of *Carnobacterium divergens* LV13 indicator cells. Zones: 1, variant 187-22; 2, Pronase E (1 mg/ml) treated UAL187 parent strain; 3, trypsin (1 mg/ml) treated UAL187 parent strain; 4, variant 187-13; 5, UAL187 parent strain; 6, catalase (100 U/ml) treated UAL187 parent strain; 7, variant 187-2; 8, variant 187-23; and 9, heat treated (62°C/30 min) UAL187 parent strain.

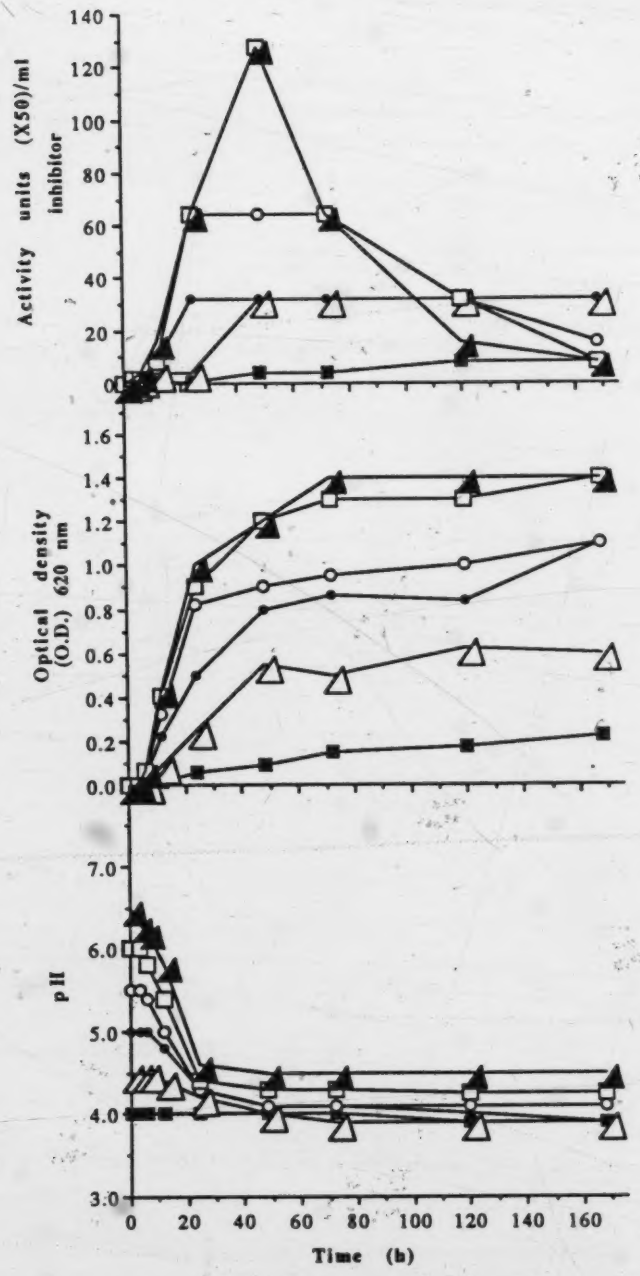

Figure 2.2. Cell density (O.D. 620 nm), amount of inhibitory activity (activity units/ml) and pH change in APT broth of initial pH 4.0 (■), 4.5 (△), 5.0 (●), 5.5 (○), 6.0 (□) and 6.5 (▲), inoculated with *Leuconostoc gelidum* UAL187 and incubated at 25°C.

each testing period. At lower pH, growth of the producer organism was slower and concentration maxima of the inhibitory substance were lower.

When *Leuc. gelidum* UAL187 was incubated at 5°C (Figure 2.3), the inhibitory substance was first detected in APT adjusted to pH 5.5 to 6.5 after incubation for 12 h, at pH 4.5 and 5.0 after 24 h, and at pH 4.0 after incubation for 72 h. Maximum production of inhibitory substance coincided with achievement of maximum population. Thereafter the concentration remained stable and eventually declined. When the organism was incubated at 1°C, for all pH levels tested, the quantity of inhibitory substance produced increased with time of incubation and it was at its highest at the end of the experiment (168 h). At the slower growth rate, production of inhibitory substance was reduced. In APT broth adjusted to pH 6.5, however, the production of inhibitory substance was detected after incubation for 12 h, at pH 5.5 and 6.0 after 24 h, and at pH 4.0, 4.5 and 5.0 after 48 h. Maximum concentration of inhibitory substance at pH 5.5 to 6.5 was detected after incubation for 120 h, whereas in APT broth adjusted to lower pH levels, the production of inhibitory substance was still occurring when the experiment was terminated at 168 h.

The killing kinetics of the inhibitory substance produced by *Leuc. gelidum* UAL187 tested against indicator strain LV13 is shown in Figure 2.4. In the presence of 100 AU of inhibitory substance per ml of APT broth the population of the indicator strain remained static for 24 h. An increase in viable count was detected after 48 h. With the same concentration of inhibitory substance in phosphate buffer (pH 6.5) the viable number of indicator cells decreased 100-fold after 48 h. However, the number of viable cells in the phosphate buffer control sample decreased approximately 10-fold. The net effect of 100 AU inhibitor/ml was to decrease the number of indicator cells 10-fold over a period of 24 to 48 h in phosphate buffer at pH 6.5. When undiluted samples were plated the inhibitory substance remained active, unless treated with protease. At dilutions greater than 1/100 the inhibitory effect was eliminated, therefore, the indicator bacteria grew without inhibition and the counts were not a true reflection of the number of cells inhibited. When cells were

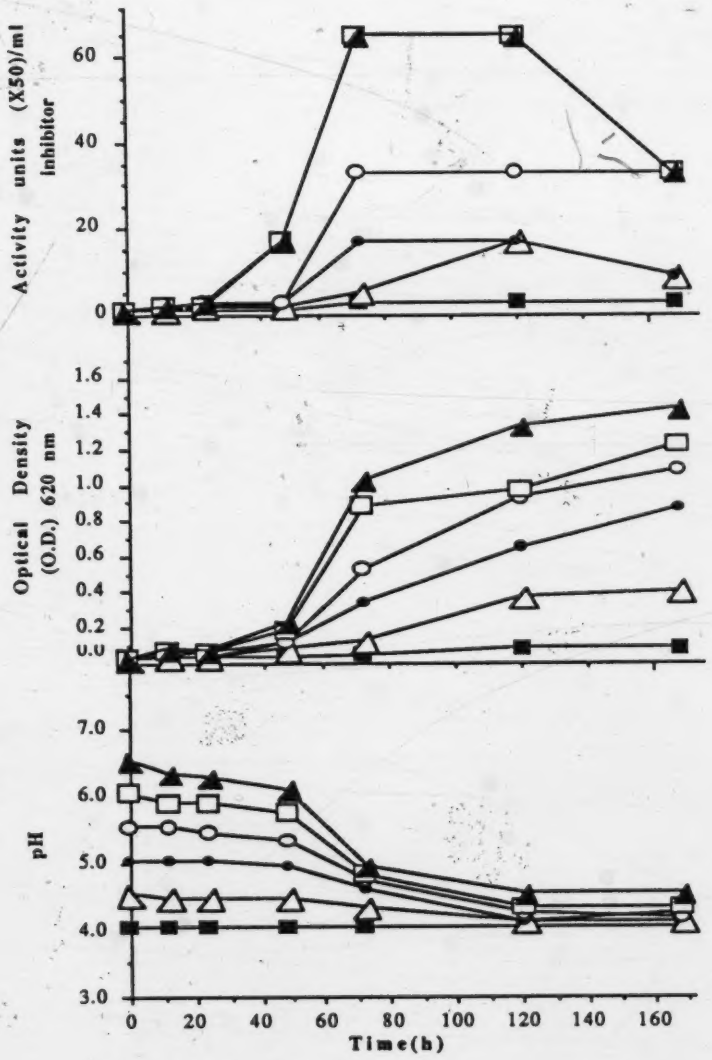

Figure 2.3. Cell density (O.D. 620 nm), amount of inhibitory activity (activity units/ml) and pH change in APT broth of initial pH 4.0 (■), 4.5 (△), 5.0 (●), 5.5 (○), 6.0 (□) and 6.5 (▲) inoculated with *Leuconostoc gelidum* UAL187 and incubated at 5°C.

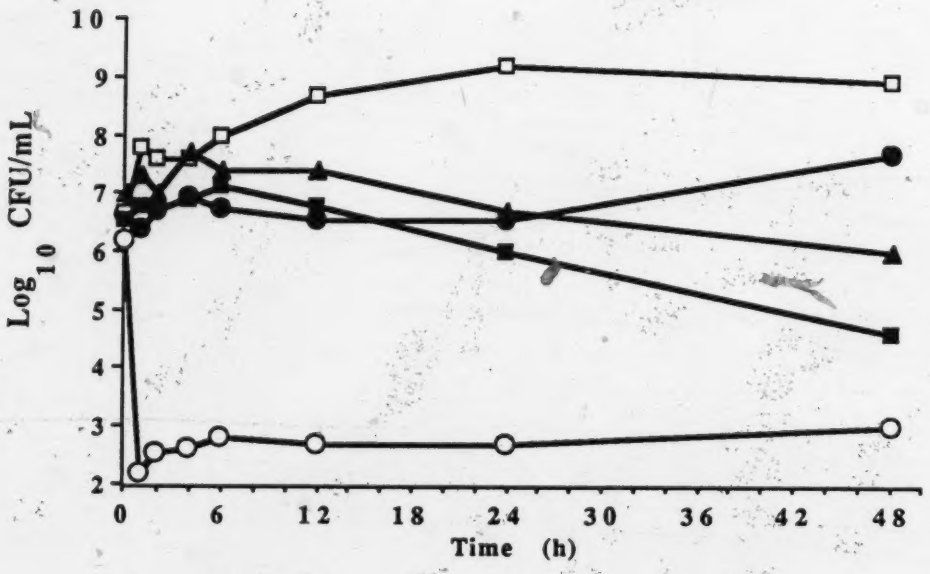

Figure 2.4. **Survivor curves of *Carnobacterium divergens* LV13 treated with antagonistic substance produced by *Leuconostoc gelidum* UAL187** when suspended in APT broth with inhibitory substance at 100 AU/ml (●), APT broth without added inhibitor (□), phosphate buffer (pH 6.5) with inhibitory substance at 100 AU/ml (■), phosphate buffer (pH 6.5) with inhibitory substance at 100 AU/ml (○ = count from undiluted sample) and phosphate buffer (pH 6.5) without added inhibitor (▲). Growth of indicator LV13 in the presence of inhibitor (100 AU/ml) and protease (1 mg/ml) was essentially the same as in APT broth without added inhibitor.

released from the effect of inhibitory substance, either by protease treatment or by dilution, the cells revived.

The plasmid profile of the parent strain was difficult to resolve (Figure 2.5). There were six distinct bands (5.0, 7.4, 7.6, 9.2, 11.7 and 14.1 MDa) of plasmid DNA separated below the chromosome band. With the method of Hintermann *et al.* (12), it was shown that the 7.4, 11.7 and 14.1 MDa bands had the same electrophoretic mobility as the open circular (OC) forms of the 5.0, 7.6 and 9.2 MDa plasmids, respectively. Therefore, there appears to be only three plasmids, i.e. 5.0, 7.6 and 9.2 MDa.

The growth of *Leuc. gelidum* UAL187 in APT broth containing 5 µg/ml of novobiocin and 0.002% SDS resulted in the selection of variants with low or reduced activity against the indicator strain LV13. Several of the strains had lost the smallest 5.0 MDa plasmid, as shown in Figure 2.5, lanes A (187-22) and C (187-2) and retained their antibacterial activity. Others had lost two plasmids, represented by the 5.0 and 7.6 MDa DNA bands and the 11.7 MDa OC form band (Figure 2.5, lane B). These variants did not produce inhibitory substance against LV13 when tested by deferred antagonism or by the spot-on-lawn technique (Figure 2.6A). Furthermore, they had lost resistance (Figure 2.6B) to the inhibitory substance produced by the parent strain and variants 187-2, -22 and -23, whereas these strains were resistant to the inhibitory substance. The phenotypes of all variant strains showed 100% homology with the parent strain *Leuc. gelidum* UAL187 described previously, except for the loss of the capacity to produce inhibitory substance and loss of resistance by strain 187-13.

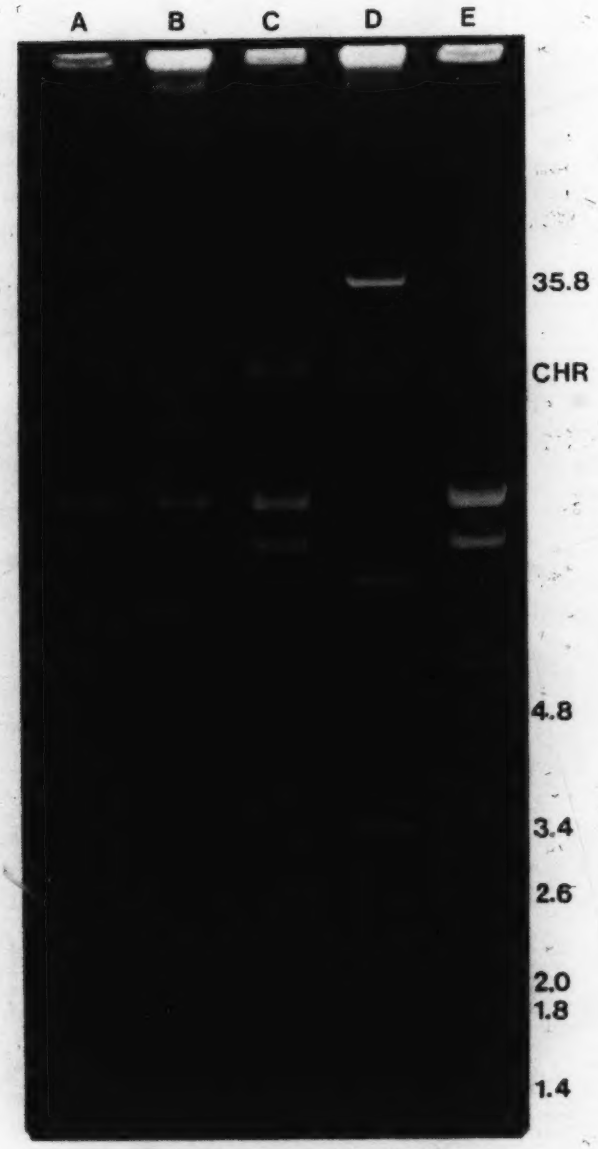

Figure 2.5. Plasmid DNA profiles of *Leuconostoc gelidum* UAL187 and variants. Lane: A, variant 187-22; B, variant 187-13; C, variant 187-2; D, *Escherichia coli* V517 (molecular mass reference standard for plasmid size); and E, UAL187 parent strain. CHR — indicates the chromosomal band.

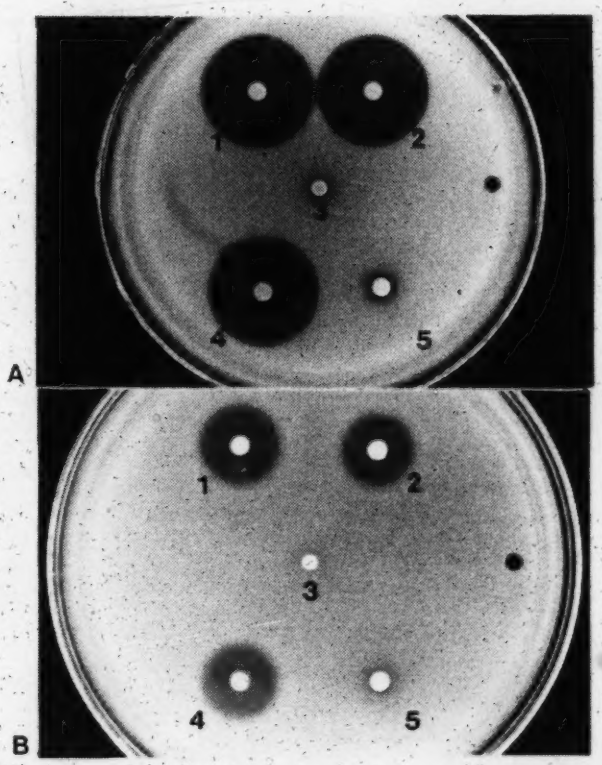

Figure 2.6. Inhibition zones produced by colonies of *Leuconostoc gelidum* UAL187 and variants.
Plate A. On a lawn of *Carnobacterium divergens* LV13 indicator cells. Zones: 1, UAL187 parent strain; 2, Variant 187-22; 3, *Carn. divergens* LV13; 4, variant 187-2; and 5, variant 187-13.
Plate B. On a lawn of variant 187-13 as indicator cells. Zones are as described for plate A.

2.4 Discussion

The organism isolated as a predominating strain of lactic acid bacteria growing in meat that had been packaged and stored in modified gas atmosphere containing elevated CO_2 was shown to be *Leuc. gelidum*. It was originally identified as *Leuc. mesenteroides* subsp. *mesenteroides* based on the description of Garvie (9). However, Shaw and Harding (23) described two new *Leuconostoc* species and with their criteria the organism was shown to be *Leuc. gelidum*, mainly because of its inability to grow at 35°C or to produce acid from galactose, as well as its ability to produce acid from amygdalin, ribose and salicin.

The organism was antagonistic to the growth of a large number of other lactic acid bacteria. The inhibitory substance was shown to be proteinaceous by protease inactivation and precipitation with ammonium sulphate. The inhibitory effect was not attributable either to pH, organic acid or hydrogen peroxide produced by the culture. The narrow spectrum of activity and proteinaceous nature of the substance indicate that it could be a bacteriocin (14). The killing kinetics, however, indicated that the substance is most likely bacteriostatic rather than bactericidal against indicator strain LV13; hence it is best described as a bacteriocin-like substance, pending further study of its antimicrobial activity.

Production of the bacteriocin-like substance by *Leuc. gelidum* UAL187 occurs early in the growth cycle of the organism, rather than as a secondary metabolite of growth. This is similar to two of the strains of meat lactics reported by Ahn and Stiles (1). Other strains only produced detectable levels of antagonistic substances by the end of the logarithmic phase of growth. Production occurs over a wide range of incubation temperatures and pH levels of the growth substrate. With such a broad range of growth conditions the organism could produce its inhibitory substance in a variety of refrigerated foods. This strain, therefore, has interesting potential as a food bacterium. The bacteriocin-like substance is relatively stable to the metabolic products of growth of the test strain especially

at low temperatures. However, there was a gradual loss of activity after 48 h at 25°C, possibly due to extracellular proteases.

The complex plasmid profile evident in this organism is not exceptional in this genus. Orberg and Sandine (19) reported as few as one or as many as seven plasmids, ranging in size from 1.9 to 65 MDa in the *Leuconostoc* strains in their study. From the genetic information it appears that production of the bacteriocin-like substance by *Leuc. gelidum* UAL187 is plasmid mediated. The specific plasmid associated with bacteriocin production has not been confirmed, but it is apparent that the smallest plasmid (5.0 MDa) is not involved. However,with the loss of the 7.6 MDa plasmid, bacteriocin production and immunity to the bacteriocin are also lost. The co-existence of bacteriocin production and immunity phenotypes with plasmids is common in the lactic acid bacteria (13, 19, 25). In some cases, the genetic determinants for bacteriocin were definitely plasmid-mediated (17). The confirmed association of bacteriocin production with plasmid-mediated genetic information requires further study.

2.5 References

1. Ahn, C., and M.E. Stiles. 1990. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. J. Appl. Bacteriol. 69:302-310.
2. Anderson, D.G., and L.L. McKay. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. 46:549-552.
3. Babel, F.J. 1977. Antibiosis by lactic culture bacteria. J. Dairy Sci. 60:815-821.
4. Barefoot, S.F., and T.R. Klaenhammer. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 45:1808-1815.
5. Daeschel, M.A., and T.R. Klaenhammer. 1985. Association of a 13.6-megadalton plasmid in *Pediococcus pentosaceus* with bacteriocin activity. Appl. Environ. Microbiol. 50:1538-1541.
6. De Klerk, H.C., and J.A. Smit. 1967. Properties of a *Lactobacillus fermenti* bacteriocin. J. Gen. Microbiol. 48:309-316.
7. DeMan, J.C., M. Rogosa, and M.E. Sharpe. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23:130-135.
8. Gawehn, K. 1984. D(-)-Lactate. In *Methods of Enzymatic Analysis*, 3rd ed. *Metabolites 1: Carbohydrates*, ed. Bergmeyer, H.U. pp. 588-592. Weinheim: Verlag Chemie.
9. Garvie, E.I. 1987. Genus Leuconostoc. In: *Bergey's Manual of Systematic Bacteriology*, Vol. 2, ed. Sneath, P.H., Mair, N.S., Sharpe, M.E., and Holt, J.G. pp. 1071-1074. Baltimore: The Williams and Wilkins Co.
10. Geis, A., J. Singh, and M. Turner. 1983. Potential of lactic streptococci to produce bacteriocin. Appl. Environ. Microbiol. 45:205-211.
11. Graham, D.C., and L.L. McKay. 1985. Plasmid DNA in strains of *Pediococcus cerevisiae* and *Pediococcus pentosaceus*. Appl. Environ. Microbiol. 50:532-534.

12. Hintermann, G., H.M. Fischer, R. Crameri, and R. Hutter. 1981. Simple procedure for distinguishing CCC, OC, and L forms of plasmid DNA by agarose gel electrophoresis. Plasmid 5:371-373.

13. Joerger, M.C., and T.R. Klaenhammer. 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Appl. Bacteriol. 167:439-446.

14. Klaenhammer, T.R. 1988. Bacteriocins of lactic acid bacteria. Biochimie 70:337-349.

15. Macrina, F.L., D.J. Kopecko, K.R. Jones, D.J. Ayers, and S.M. McCowen. 1978. A multiple plasmid containing *Escherichia coli* strain: Convenient source of size reference plasmid molecules. Plasmid 1:417-420.

16. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory, Cold Spring Harbor.

17. Muriana, P.M., and T.R. Klaenhammer. 1987. Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. Appl. Environ. Microbiol. 53:553-560.

18. Noll, F. 1984. L(+)- Lactate. In *Methods of Enzymatic Analysis*, 3rd ed. *Metabolites 1: Carbohydrates*, ed. Bergmeyer, H.U. pp. 582-588, Weinheim: Verlag Chemie.

19. Orberg, P.K., and W.E. Sandine. 1984. Common occurrence of plasmid DNA and vancomycin resistance in *Leuconostoc* spp. Appl. Environ. Microbiol. 48:1129-1133.

20. Schillinger, U., and F.-K. Lücke. 1987. Identification of lactobacilli from meat and meat products. Food Microbiol. 4:199-208.

21. Schillinger, U., and F.-K. Lücke. 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. 55:1901-1906.

22. Shaw, B.G., and C.D. Harding. 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. J. Appl. Bacteriol. 56:25-40.

23. Shaw, B.G., and C.D. Harding. 1989. *Leuconostoc gelidum* sp. nov. and *Leuconostoc carnosum* sp. nov. from chill-stored meats. Internat. J. Syst. Bacteriol.39:217-233.

24. Tagg, J.R., A.S. Dajani and L.W. Wannamaker. 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. 40:722-756.

25. Upreti, G.C., and R.D. Hindsdill. 1975. Production and mode of action of lactocin 27: Bacteriocin from heterofermentative *Lactobacillus*. Antimicrob. Agents Chemother. 7:145-149.

3. Purification and Characterization of Leucocin A-UAL187, a Bacteriocin from *Leuconostoc gelidum*[1]

3.1 Introduction

Bacteriocins are antimicrobial peptides or proteins formed by bacteria (33). The potential applications of bacteriocin-producing lactic acid bacteria in food processing and preservation has recently stimulated considerable interest in the characterization of these substances (20, 23). However, maintaining activity during isolation and purification has proved difficult, and the full or partial amino acid sequences of only five such bacteriocins have been reported. The most extensively studied is nisin A (1, 2, 6, 11, 29), a post-translationally modified bacteriocin from *Lactococcus lactis* subsp. *lactis* consisting of 34 amino acids. Nisin has been approved for use as a preservative in foods in over 45 countries (7, 15). It is ribosomally synthesized (5, 8), and is one of a group of "lantibiotics" e.g. actagardine (19), gallidermin (18), epidermin (30), lanthiopeptin (34), ancovenin (35) that possess lanthionine or methyllanthionine containing rings resulting from attack of cysteine sulphydryl groups on dehydroalanine or dehydrobutyrine residues (derived from serine or threonine). Partial characterization of lactacin 481 from *L. lactis* shows that it also contains lanthionine rings, but the full sequence has not yet been published (27). In contrast, lactocin S from *Lactobacillus sake* (24) and lactacin F produced by *Lactobacillus acidophilus* (25) are peptides containing 54 and 57 amino acids, respectively, that do not contain modified amino acids. A third group of bacteriocins are considerably larger proteins. Helveticin J generated by *Lactobacillus helveticus* has a molecular weight of 37,511 based on its gene sequence (17). Similarly, caseicin 80 from *Lactobacillus casei* is estimated to be a 40,000-42,000 dalton protein based on gel filtration experiments, but the amino acid sequence is still unknown (28).

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[1] *A version of this chapter has been submitted for publication in the Journal of Bacteriology by:* Hastings, J.W., M. Sailer, K. Johnson, J.C. Vederas and M.E. Stiles. 1991.

It is well established that various *Leuconostoc* species display antimicrobial activity against other lactic acid bacteria (20, 26), but until recently little was known about the chemical nature of the active compounds. Harding and Shaw reported that a strain of *Leuconostoc gelidum* inhibited a wide spectrum of lactic acid bacteria, meat spoilage bacteria, and food-related human pathogens, including *Listeria monocytogenes* (12). We recently described a bacteriocin-like substance produced by a different strain of *Leuc. gelidum*, isolated from meat (13; see Chapter 2). The active compound was formed at temperatures ranging from 1 to 25°C and at pH levels from 4.0 to 6.5. This material is inactivated by protease but not by catalase treatment, and shows activity against a wide range of lactic acid bacteria as well as against some strains of bacteria that are associated with foodborne illness. We now report the isolation, purification, structure elucidation, and properties of this new bacteriocin from *Leuc. gelidum* UAL 187. This compound is designated as leucocin A - UAL 187 according to the nomenclature described by Tagg *et al.* (33). Its molecular weight, amino acid analysis, amino-terminal sequencing, and mass spectral analysis are described and compared with the putative amino acid sequence of the ribosomally synthesized pre-peptide.

3.2 Materials and methods

3.2.1 Cultures and media

The bacteriocin producer *Leuc. gelidum* UAL 187, the indicator strain *Carnobacterium divergens* LV13 and the conditions for maintenance and growth of these cultures have been described (13). A semi-defined medium was used for production of Leucocin A-UAL 187 which included (per liter of solution): casamino acids (Difco Laboratories, Detroit, MI), 15 g; yeast extract (BBL Microbiology Products, Cockeysville, MD), 5 g; D-glucose (BDH Chemicals Ltd., Poole, England), 20 g; dipotassium phosphate (J.T. Baker Chemical Co., Phillipsburg, NJ), 2 g; Tween 80 (Difco), 1 ml; di-ammonium citrate

(BDH), 2 g; magnesium sulphate (Anachemia, Champlain, NY), 0.1 g; and manganous sulphate (BDH), 0.05 g. This medium is subsequently referred to as CAA medium.

3.2.2 Production of leucocin A-UAL 187

An inoculum of 100 ml of overnight culture of *Leuc. gelidum* UAL 187 was added to 5 liters of CAA medium. A Chemcadet pH controller (Cole/Parmer, Chicago, Illinois) maintained the pH at 6.0 with 1N sodium hydroxide. The temperature was kept at 22-23°C, and the culture was gently stirred under N_2 atmosphere (40 ml/min). After about 24 hours there was no further pH change, and the cells were harvested by centrifugation at 6,000 x g for 15 min at 4°C.

3.2.3 Purification of leucocin A-UAL 187 from supernatant

The active substance in one half of the supernatant (2.5 l) was precipitated with 70% ammonium sulphate and the other half was precipitated by lowering the pH to 2.5 using 12 N HCl . The precipitates from both ammonium sulphate and pH treatments were dissolved in 6 M urea (Fisher), 10 mM glycine-HCl (pH 2.5) and loaded onto a 6 x 25 cm Amberlite XAD-2 column (Pharmacia, Uppsala, Sweden). The column was washed with 0.1% TFA (trifluoroacetic acid) (1.5 l), 25% ethanol/0.1% TFA (1.0 l) and 45% ethanol/0.1% TFA (1.0 l). Leucocin was eluted with 75% ethanol/0.1% TFA (2.0 l). Active fractions (15 ml) were pooled and lyophilized after the ethanol was evaporated using a rotary evaporator (≤ 30°C). The residue was resuspended in 0.1% TFA and loaded onto a Sephadex G-25 (Pharmacia) column (2.5 x 45 cm) equilibrated with 0.1% TFA. Elution was monitored at 220 nm and all fractions (4 ml) were assayed for bacteriocin activity. Only fractions containing more than 100 arbitrary activity units (AU, determined against *Carnobacterium divergens* LV13) of bacteriocin per ml were pooled and lyophilized. The dry sample was dissolved in 0.1% TFA and purified on a BioRad HPLC instrument using a C-18 column (8 x 100 mm Waters µ-Bondapak, 5 µ particle size, 125 Å). Leucocin was isocratically eluted (1.65 ml/min) using 35% acetonitrile / 0.15% TFA / H_2O. Elution was

monitored at 220 nm. Activity and total protein determinations were performed at each stage. The activity assay was previously described (13). Total protein content was determined using the method of Lowry *et al.* (21) as modified by Markwell *et al.* (22).

3.2.4 Tests for stability of leucocin

The effect of pH, temperature, degradative enzymes and selected solvents on the activity of leucocin (400 AU/ml) was determined. Pure and crude (after pH precipitation) samples of bacteriocin were suspended in 50 mM glycine-HCl pH 2.0 and 3.0; 50 mM sodium acetate pH 4.0 and 5.0; 50 mM sodium citrate pH 6.0; 60 mM Tris-HCl pH 7.0 and 8.0; 50 mM glycine-NaOH pH 9.0 and 10.0. Leucocin samples in buffer solutions (200 µl) were placed on ice and at 25°C. Activity was determined immediately and after holding for 2 and 24 h. One sample in each buffer was placed in boiling water for 20 min and cooled to room temperature for determination of residual activity.

Samples of pure and crude leucocin suspended in the buffers of pH 2.0, 6.0 and 10.0 containing 10 mg/ml bovine serum albumin (BSA, Sigma) per ml or 0.5% dithiothreitol (DTT, Sigma) were treated as described above. DTT samples were tested at 0°C only.

Enzymes (1 mg/ml) were added to purified leucocin (400 AU/ml) as follows: Proteases types I (crude, from bovine pancreas), IV (pure, from *Streptomyces caespitosus*, VIII (Subtilisin Carlsberg), X (Thermolysin) and XIV (Pronase E) (Sigma) in 50 mM Tris-HCl pH 7.5, and type XIII (Aspergillopeptidase Molsin) in 50 mM acetate buffer pH 4.0; trypsin (Sigma) in 50 mM Tris-HCl pH 7.5; and α- and ß-chymotrypsin in 50 mM Tris-HCl 10 mM $CaCl_2$, pH 7.5; pepsin in 10 mM citrate pH 6.0; papain and lysozyme in 50 mM Tris-HCl pH 7.5; lipase and phospholipase C in 50 mM Tris-HCl and 10 mM $CaCl_2$ pH 7.0. Activity was determined immediately.

Crude leucocin (ammonium sulphate concentrate, dissolved in 6 M urea/50 mM glycine-HCl, pH 2.0) was suspended in various organic solvents to give a final concentra-

tion of 6,400 AU of bacteriocin per ml. The following solvents diluted in 50 mM glycine at pH 2.0 were used: 50% ethanol (BDH); 70% ethanol (BDH); 50% acetonitrile; 0.15% trifluoroacetic acid (Sigma); 50% tetrahydrofluoride (Caledon Laboratories Ltd., Georgetown, ON); 50% isopropanol (BDH); 50% acetone (BDH) and 50% methanol (BDH). Activity was determined after 2 h at room temperature. Solvents were evaporated using a rotary evaporator, and samples were lyophilized and resuspended in 25 mM glycine-HCl pH 2.0 buffer to determine activity.

3.2.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The leucocin preparations were examined on a 20% polyacrylamide gel in 0.5% SDS and 0.33 M Tris, pH 6.8. Electrophoresis was done at 25 mA for the stacking gel and 50 mA for the separating gel. After electrophoresis, gels were either fixed in 5% formaldehyde (w/v) for 1 h and stained with Coomassie blue (BioRad, Richmond, CA) or assayed for antimicrobial activity using the method of Barefoot and Klaenhammer (3).

3.2.6 Determination of amino acid content and sequence of leucocin A-UAL 187

Purified leucocin was derivatized with phenylisothiocyanate (PITC) on an Applied Biosystems 420A derivatizer system and separated on an Applied Biosystems HPLC model 130A using a C-18 column by M. Carpenter, Dept. of Biochemistry, Univ. of Alberta. Data was recorded and analyzed on an Applied Biosystems 920A data analyzer system. The amino terminal sequence of leucocin was determined by automated Edman degradation using an Applied Biosystems model 470A gas-phase sequencer with on-line PTH (phenylthiodantoin)-derivative identification by an Applied Biosystems HPLC model 120A.

3.2.7 Cleavage of leucocin A-UAL 187 by trypsin

A sample of 0.8 mg of leucocin in 50 mM pH 6.0 citrate buffer (1.0 ml) was treated with 2.0 mg of trypsin (Sigma) in the same buffer (1.0 ml) for 2 h at 24°C. Trypsin was removed by chromatography on Sephadex G-25 (2.5 x 2.5 cm, 0.1% TFA in water) and

the other fractions were combined and lyophilized. The residue was dissolved in 0.1% TFA in water (1.5 ml) and separated by HPLC on a C-18 column (8 x 100 mm Waters µ-Bondapak, 5m particle size, 125 Å) with a flow rate of 1.65 ml/min using 35% acetonitrile / 0.1% TFA / H_2O. Four fractions were collected and two of these were analyzed by mass spectrometry (see below).

3.2.8 Mass spectrometry

All mass spectrometry was performed by K. Johnson of Finnigan MAT (San José, CA) on a Finnigan MAT TSQ 700 triple quadrupole instrument fitted with an electrospray ionization source from Analytica (Bradford, CT).

3.2.9 Cleavage of the disulphide bond in leucocin A-UAL 187 and derivatization with iodoacetic acid

Leucocin was reduced with ß-mercaptoethanol, derivatized with iodoacetic acid, and purified by RP-HPLC by Dr. M. Sailer, Dept. of Chemistry, Univ. of Alberta.

3.3 Results

3.3.1 Purification of leucocin A-UAL 187

Before commencing the purification of leucocin A-UAL 187 it was established that the bacteriocin was stable below pH 3.0 and unstable above pH 7.0. Care was taken to use procedures in which a low pH was maintained. The stability of the bacteriocin in various solvents was also tested to determine which solvents could be employed in the separation process. Of the organic solvents tested, only 50% methanol had a detrimental effect on the activity of the bacteriocin. Ethanol, trifluoroacetic acid and acetonitrile were used in the separation process. The purification stages of leucocin are shown in Table 3.1. The recovery of bacteriocin after 70% ammonium sulphate and pH 2.5 precipitation was similar. Each procedure recovered approximately 30 million AU of the bacteriocin. The precipitates were not soluble in 50 mM glycine-HCl, pH 2.5. Addition of 6 M urea (final

Table 3.1. Purification of leucocin A-UAL 187.

Purification Stage	Vol (ml)	Leucocin activity (AU/ml)[a]	Total leucocin activity (AU)	Amount of protein (mg/ml)[b]	Specific activity (AU/mg)	Activity recovery (%)	Fold purification
Culture supernatant	5000	1.3×10^4	6.4×10^7	3.2	4×10^3	100	1
Combined ammonium sulphate and pH 2.5 concentrate	18	3.3×10^6	6.0×10^7	9.5	3.5×10^5	94	87
Pooled Amberlite XAD-2 fractions (0.1 % TFA/EtOH)	900	6.4×10^4	5.8×10^7	0.12	5.3×10^5	90	133
Pooled Sephadex G-25 fractions (0.1 % TFA)	420	1.0×10^5	4.3×10^7	0.06	1.7×10^6	67	428
RP-HPLC (35%ACN/0.1% TFA) separation, lyophilized, resusp. in 0.1% TFA	1.8	2.1×10^7	3.7×10^7	1.14	1.8×10^7	58	4,500

a AU, Activity units determined against *Carnobacterium divergens* LV13.
b Determined by method of Lowry *et al.* (21) as modified by Markwell *et al.* (22) using Nisin-A (Aplin and Barrett, Trowbridge, England)) as a standard.

concentration) to the buffer was necessary to solubilize the precipitate. The crude concentrate was applied to an amberlite XAD-2 column (2.5 x 20 cm) 0.1% TFA to bind the bacteriocin. The column was washed with 0.1% TFA, and TFA-ethanol solutions. The bacteriocin was eluted with 75% ethanol/0.1% TFA. Only 4% of total starting activity was lost in this step. The pooled, active fractions were evaporated, lyophilized, resuspended in 0.1% TFA and loaded onto a Sephadex G-25 column (2.5 x 47 cm) in 0.1% TFA. Figure 3.1 shows the elution spectrum from the Sephadex column. Most of the bacteriocin was eluted in a single band, however, some activity was detected in all subsequent fractions. Only fractions containing more than 100 AU/ml were pooled.

To obtain pure leucocin using an HPLC C-18 column, several gradient elutions were tried. However, isocratic elution with 35% acetonitrile/0.15% TFA gave the best separation (Figure 3.2). The HPLC-purified sample was initially visualized on a 20% SDS-PAGE gel (Figure 3.3). Resolution of the small peptides was difficult. They appeared as faint, diffuse bands. Resolution was improved by increasing the amount of SDS from 0.1% to 0.5% and the amount of Tris to 0.33M (final concentration). The boldness of the band was improved by fixing the gel in 5% formaldehyde before staining (9). The estimation of molecular weight of the bacteriocin by gel electrophoresis was between 2,500 and 3,000 daltons. Mass spectrometry, however, determined the exact mass of leucocin to be 3930.4 daltons.

3.3.2 Stability of leucocin A-UAL 187

From the data in Table 3.2, it can be seen that the crude isolate of leucocin is stable at pH 2.0 and 3.0, even after boiling samples for 20 min. Activity loss was detected at pH 5.0 and above. This was most severe in samples above pH 8.0 after 24 h on ice. Activity of the pure sample was lost rapidly at all pH levels and temperatures tested. However, when a similar experiment was done with an increased concentration of leucocin (Table 3.3), loss of activity was slowed and the bacteriocin could be boiled in buffer at pH 2.0

Table 3.2. The effect of pH and temperature on the antimicrobial activity of RP-HPLC purified and crude (ammonium sulphate concentrate) leucocin A-UAL 187.

Leucocin preparation	Pure[a]				Crude		
Temperature	1°C		1°C		25°C		100°C
Time (h)	2	24	2	24	2	24	0.33
	AU/ml[b]						
pH 2.0	200	<100	400	400	400	400	400
pH 3.0	200	<100	400	400	400	400	400
pH 4.0	200	<100	400	400	400	400	200
pH 5.0	200	<100	400	200	400	400	200
pH 6.0	200	<100	200	200	400	400	200
pH 7.0	200	<100	200	100	200	200	200
pH 8.0	200	<100	200	<100	200	100	100
pH 9.0	100	<100	100	<100	200	100	<100
pH 10.0	100	<100	100	<100	100	<100	<100

a No leucocin activity was detected in RP-HPLC purified samples incubated at 25°C for 2 and 24 hours.

b Activity units/ml after 2 hours incubation at 1°C and 25°C and after 20 min at 100°C. Starting activity for all tubes was 400 AU/ml.

Table 3.3. The effect of bovine serum albumin (BSA) and dithiothreitol (DTT) on the heat and pH stability of RP-HPLC purified leucocin A-UAL 187.

	Leucocin			Leucocin + BSA			Leucocin + DTT		
Temperature	1°C	25°C	100°C	1°C	25°C	100°C	1°C	25°C	100°C
	AU/ml[a]								
pH 2.0	200	100	100	3,200	3,200	3,200	100	100	<100
pH 6.0	100	<100	<100	3,200	3,200	200	100	ND[b]	ND
pH 10.0	100	<100	<100	3,200	1,600	<100	100	ND	ND

a Activity units/ml after 2 hours incubation at 1°C and 25°C and after 20 min at 100°C. Initial activity for all samples was 800 AU/ml. The addition of BSA caused an apparent 4-fold increase in activity.

b ND, not determined.

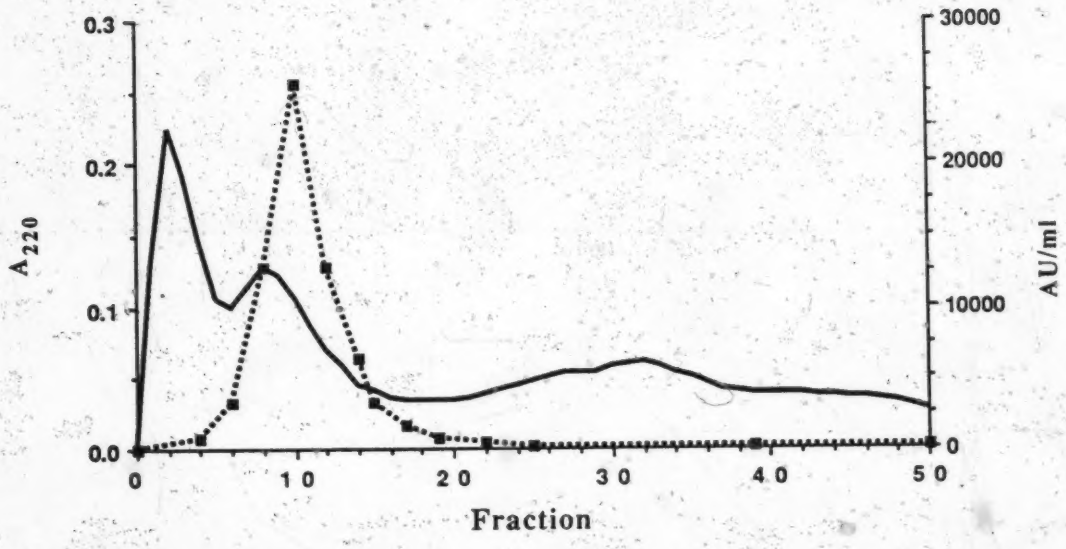

Figure 3.1. Chromatography of leucocin A-UAL 187 on Sephadex G-25 equilibrated with 0.1% TFA. A 5 ml sample (3.2 million activity units per ml in 0.1% TFA) was applied to the column. The eluent was monitored at 220 nm and fractions (4 ml) were collected and assayed for leucocin activity. Symbols: — Absorbance 220 nm; ■ Leucocin activity.

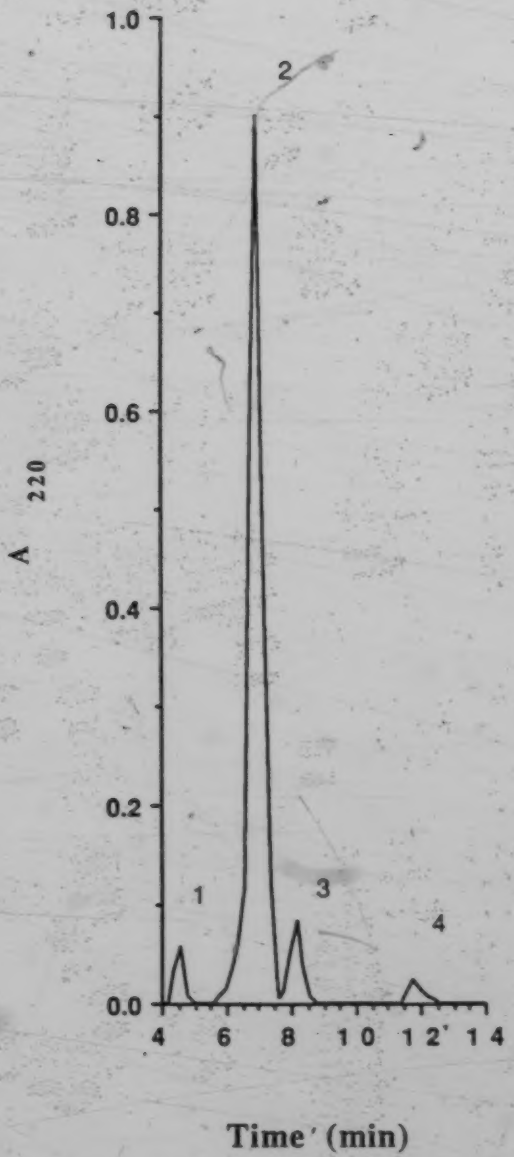

Figure 3.2. **Elution spectrum of leucocin A-UAL 187 from RP-HPLC C-18 column (Waters).** A 50 µl sample of leucocin (240,000 activity units) in 0.1% TFA was applied to the column. Isocratic elution was performed with 35% acetonitrile in 0.1% TFA and monitored at 220 nm. Four fractions (corresponding to peaks 1-4) were collected, the acetonitrile evaporated and activity determined. Fraction 2 contained 99% of all activity.

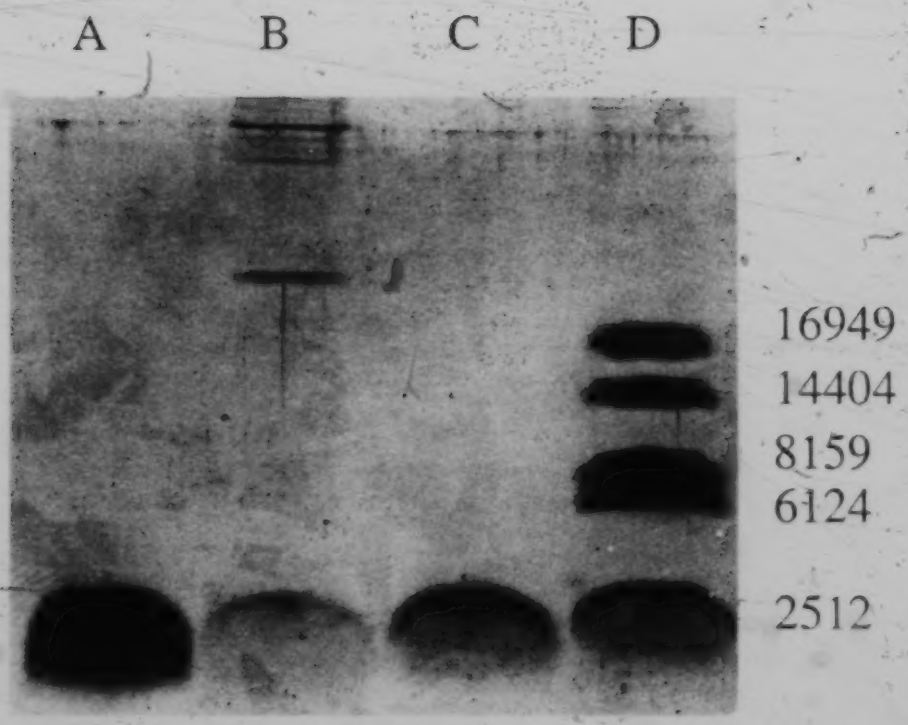

Figure 3.3. Polyacrylamide gel (20%) electrophoresis of leucocin A-UAL 187 preparations in the presence of 0.5% SDS and 0.33M Tris pH 6.8, fixed with 5% formaldehyde and stained with Coomassie blue. Lane A, RP-HPLC purified leucocin; Lane B, crude leucocin (after pH precipitation); Lane C, Nisin (3353 daltons) (Aplin and Barrett, Trowbridge, England); and Lane D, low molecular weight protein standards (cyanogen bromide cleavage of horse myoglobin): 16,949, 14,404, 8,159, 6,124, and 2,512 daltons (Fluka, Biochemika).

and some activity was retained. Adding BSA to the pure bacteriocin (Table 3.3) gave recovery of activity similar to that observed with the crude sample. In the experiment to determine whether BSA reversed the denaturation process or enhanced activity, the activity after 2 h a 25°C in the sample containing BSA was unchanged at 3,200, but only 400 AU/ml was detected in the sample without BSA. Samples with BSA showed a 4-fold increase in activity units. The sample without BSA was divided into two aliquots and BSA was added to one and activity was determined immediately. The sample with BSA showed 1,600 AU/ml which is equivalent to half of the initial activity. The loss of activity in the presence of DTT was the same or greater than that observed in the control.

Treatment with proteases I, IV, VIII, X, XIII and XIV, trypsin, α- and ß-chymotrypsin, pepsin and papain caused complete loss of activity of purified leucocin A- UAL 187. However, lysozyme, lipase and phospholipase did not cause any loss in activity compared with the control.

3.3.3 Amino acid analysis and sequence of leucocin A-UAL 187

The N-amino acid terminal sequence identified 13 amino acids shown in Figure 3.4. After this point the yield of PTH derivatized amino acid residues decreased sharply to undetectable levels. The putative sequence shown in Figure 3.4 is derived from double-stranded nucleotide sequencing (31) of the *lcn* gene (14; see Chapter 4). The data indicates a 37 amino acid bacteriocin and the amino acid analysis of purified leucocin (Table 3.4) confirms the putative amino acid compositional data.

3.3.4 Mass spectrometry of pure leucocin A-UAL 187

Mass spectral measurements on samples of pure leucocin using electrospray ionization (10) and a triple quadrupole spectrometer showed that the average molecular weight is 3930.4 ± 0.4. The nominal mass of the peptide derived from the putative (nucleotide-derived) amino acid sequence is 3932.3. Leucocin appears to have no post-translational modifications except for a disulfide bridge between the cysteine residues at amino acids 9

Table 3.4. Amino acid composition of leucocin A-UAL 187 determined by acid hydrolysis, derivatization with phenylisothiocyanate (PITC) and separation by HPLC.

Amino Acids	Abbreviations for Amino Acids	PMOL	Number of Residues in Sequence[a]
Asparagine[b]	Asn	416	4
Glutamic acid	Glu	188	1
Serine	Ser	247	3
Glycine	Gly	913	8
Histidine	His	243	2
Arginine	Arg	120	1
Threonine	Thr	115	1
Alanine	Ala	353	3
Tyrosine	Tyr	192	2
Valine	Val	337	3
Leucine	Leu	137	1
Phenylalanine	Phe	231	2
Lysine	Lys	195	2
Tryptophan	Trp	ND	2
Cysteine	Cys	ND	2

[a] Determined from DNA sequence data (see Figure 3.4).
[b] Asparagine inferred from DNA sequence data.

A. N-terminal amino acid sequence

H_2N - Lys - Tyr - Tyr - Gly - Asn - Gly - Val - His - Cys - Thr - Lys - Ser - Gly .....

B. Deduced amino acid sequence

S - S

H_2N - Lys - Tyr - Tyr - Gly - Asn - Gly -Val - His - Cys - Thr - Lys - Ser - Gly - Cys -

Ser - Val - Asn - Trp - Gly - Glu - Ala - Phe - Ser - Ala - Gly - Val - His - Arg -

Leu - Ala - Asn - Gly - Gly - Asn - Gly - Phe - Trp - COOH

Figure 3.4. Amino acid sequence of leucocin A-UAL 187 determined by NH_2 terminal amino acid sequencing and deduced from the nucleotide sequence of the leucocin gene (see Chapter 4).

and 14. Digestion of pure leucocin with crude trypsin followed by HPLC separation revealed a series of four fragments. None of these tryptic fragments of leucocin displayed antibacterial activity. Two of the fragments were analyzed by mass spectrometry in the same fashion to further validate the sequence. One of these peptides is Lys-Tyr-Tyr (average molecular weight of 472.5 with observed MH^+ 473.3) arising from cleavage at the N-terminus. The other tryptic peptide fragment is Tyr-Tyr-Gly-Asn-Gly-Val-His (average molecular weight of 808.3 with observed MH^+ 809.0). Its mass spectrum also contained the doubly charged ion at 405.0 ($[M+2H]^{+2}$). The daughter spectra of both tryptic peptides support the proposed sequence.

3.3.5 Derivatization of leucocin A-UAL 187

Reduction of the disulphide linkage of leucocin with ß-mercaptoethanol followed by derivatization with iodoacetic acid generated a modified derivative that was easily separated from leucocin by HPLC. The reduced sample of leucocin had a retention time of 7.0 min and the carboxymethylated sample had a retention time of 7.6 min. Both samples displayed antibacterial activity at levels approximately the same as the parent leucocin.

3.4 Discussion

This study confirmed that leucocin A-UAL 187 is a small peptide of molecular weight 3930.4 daltons, determined by FAB-mass spectrometry. Leucocin contains 37 amino acids and belongs to a newly discovered class of bacteriocins (24, 25) that do not contain lanthionine residues. Initial experiments (data not shown) showed that leucocin A-UAL 187 is retained when bacteriocin-containing fluids are filtered through a 10,000 MW Amicon filter. Sizing experiments using filter membranes and gel filtration should be interpreted with caution, especially under nondissociating conditions. SDS-PAGE gel electrophoresis only afforded an approximation of the molecular weight because pure nisin (MW 3,353) ran in advance of the 2,512 fragment of the Fluka standards (Figure 3.3).

Leucocin was purified with a yield of 2.06 mg from 5 l of CAA growth medium. This represents a significantly higher yield than previously obtained when purifying non-lantibiotic bacteriocins (3, 16, 25). Ion exchange chromatography, dialysis and high pH conditions were avoided as they resulted in large losses in activity, similar to those reported in other studies (3, 4, 16). Keeping the pH low and using only precipitation, gel filtration and hydrophobic interactions resulted in a good yield. Similar yields were obtained when these types of procedures were used for the isolation of lantibiotics (18, 30).

In the early stages of the purification process, the bacteriocin aggregated with larger proteins in the supernatant fluids. This phenomenon was previously reported by Barefoot and Klaenhammer (3) and Bhunia *et al.* (4). This may confuse molecular weight determinations (4). Ammonium sulphate and pH precipitations yielded bacteriocin-containing protein aggregates that were not soluble in buffer alone. The addition of urea allowed dissociation of the bacteriocin from the other proteins and, upon elution from the Amberlite column, dissociating conditions were no longer necessary.

Sephadex G-25 chromatography step resulted in the largest loss of activity. This was unexpected because gel filtration caused only marginal loss of activity in other studies (3, 4, 30). This may be explained by the fact that only the fractions where the bacteriocin was concentrated were pooled. All subsequent fractions, however, showed some activity indicating some interaction between leucocin A-UAL 187 and the column matrix.

The stability of leucocin at low pH and its instability above pH 7.0 was similar to that found with other bacteriocins of low MW, such as Nisin-A and Pediocin AcH (4, 15). The prevalence of mainly basic and hydrophobic residues may account for this. Adding BSA to leucocin stabilized the pure form at low pH. The presence of BSA also affected the activity assay and showed a *ca.* 4-fold increase in activity units compared with the control. BSA may prevent denaturation of leucocin during the time between spotting of the bacteriocin and growth of the indicator. Leucocin was previously shown to be proteinaceous by virtue of its inactivation by protease and trypsin (13). The purified leucocin is

inactivated by many types of proteases but it is not inactivated by treatment with chloroform (13), lipase and phospholipase, suggesting the absence of lipid or phospholipid moieties.

Leucocin contains 37 amino acids. The first twelve residues deduced by amino-terminal sequencing were confirmed by the DNA sequence. The molecular weight calculated from the putative amino acid sequence deduced from the DNA code is 3932.3 daltons. This is 2 daltons greater than the molecular weight determined by FAB-Mass spectrometry. This indicates that there could be a loss of 2 protons in the maturation of the prepeptide. The formation of a disulfide bridge between the two cysteine residues could explain this. Derivatization of leucocin to prevent the formation of the disulphide bridge resulted, surprisingly, in no loss of activity in the derivatized leucocin. The significance of the structure of leucocin relative to its mode of activity is still undetermined.

3.5 Acknowledgements

The amino acid analysis and sequencing was done by M. Carpenter, Department of Biochemistry, University of Alberta. The mass spectrometry was done by Kenneth Johnson at Finnigan MAT, San José, California. The derivatization of leucocin A-UAL 187 was performed by Dr. M. Sailer, Dept. of Chemistry, Univ. of Alberta. The purification process was done in collaboration with Dr. Sailer.

3.6 References

1. Bailey, F.J., and A. Hurst. 1971. Preparation of a highly active form of nisin from *Streptococccus lactis*. Can. J. Microbiol. 17:61-67.
2. Barber, M., G.J. Elliot, R.S. Bordoli, B.N. Green, and B.W. Bycroft. 1988. Confirmation of the structure of nisin and its major degradation product by FAB-MS and FAB-MS/MS. Experientia 44:266-270.
3. Barefoot, S.F., and T.R. Klaenhammer. 1984. Purification and characterization of the *Lactobacillus acidophilus* bacteriocin Lactacin B. Antimicrob. Agents Chemother. 26:328-334.
4. Bhunia, A.K., M.C. Johnson, and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. J. Appl. Bacteriol. 65:261-268.
5. Buchman, G.W., S. Banerjee, and J.N. Hansen. 1988. Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. J. Biol. Chem. 263:16260-16266.
6. Chan, W.C., L.-Y. Lian, B.W. Bycroft, and G.C.K. Roberts. 1989. Confirmation of the structure of nisin by complete ^{1}H Nmr resonance assignment in aqueous and dimethyl sulphoxide solution. J. Chem. Soc. Perkin Trans I:2359-2367.
7. Delves-Broughton, J. 1990. Nisin and its uses as a food preservative. Food Technol. 44 (11):100, 102, 104, 106, 108, 111, 112, 117.
8. Dodd, H.M., N. Horn, and M.J. Gasson. 1990. Analysis of the genetic determinant for production of the peptide antibiotic nisin. J. Gen. Microbiol. 136:555-566.
9. Dunn, M.J. 1989. Electrophoretic analysis methods, p. 33. *In*: Protein Purification Methods, A Practical Approach. E.L.V. Harris and S. Angal (ed.) IRL Press, Oxford.
10. Fenn, J.B., M. Mann, C.K. Meng, S.F. Wong, and C.M. Whitehouse. 1989. Electrospray ionization for mass spectrometry of large biomolecules. Science 246:64-71.

11. Gross, E., and J.L. Morell. 1971. The structure of nisin. J. Amer. Chem. Soc. 93:4634-4635.

12. Harding, C.D., and B.G. Shaw. 1990. Antimicrobial activity of *Leuconostoc gelidum* against closely related species and *Listeria monocytogenes*. J. Appl. Bacteriol. 69:648-654.

13. Hastings, J.W., and M.E. Stiles. 1991. Antibiosis of *Leuconostoc gelidum* isolated from meat. J. Appl. Bacteriol. 70:127-134.

14. Hastings, J.W., K.L. Roy, and M.E. Stiles. 1991. Cloning, organization and sequencing of a *Leuconostoc* bacteriocin operon. J. Bacteriol. (in press).

15. Hurst, A. 1981. Nisin. Adv. Appl. Microbiol. 27:85-123.

16. Joerger, M.C., and T.R. Klaenhammer. 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Bacteriol. 167:439-446.

17. Joerger, M.C., and T.R. Klaenhammer. 1990. Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. J. Bacteriol. 172:6339-6347.

18. Kellner, R., G. Jung, T. Hörner, H. Zähner, N. Schnell, K.-D. Entian, and F. Götz. 1988. Gallidermin: a new lanthionine-containing polypeptide antibiotic. Eur. J. Biochem. 177:53-59.

19. Kettenring, J.K., A. Malabarba, K. Vékey, and B. Cavalleri. 1990. Sequence determination of actagardine, a novel lantibiotic, by homonuclear 2D NMR spectroscopy. J. Antibiot. 43:1082-1088.

20. Klaenhammer, T.R. 1988. Bacteriocins of lactic acid bacteria. Biochimie 70:337-349.

21. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:256-275.

22. Markwell, M.K., S.M. Haas, L.L. Bieber, and N.E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87:206-210.

23. McKay, L.L., and K.A. Baldwin. 1990. Applications for biotechnology: present and future improvements in lactic acid bacteria. FEMS Microbiol. Rev. 87:3-14.

24. Mortvedt, C.I., J. Nissen-Meyer, and I.F. Nes. 1990. Purification of a bacteriocin from *Lactobacillus sake* and its association to a 50 KB plasmid. FEMS Microbiol. Rev. 87:P92 (abstract).

25. Muriana, P.M., and T.R. Klaenhammer. 1991. Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. Appl. Environ. Microbiol. 57:114-121.

26. Orberg, P.K., and W.E. Sandine. 1984. Common occurrence of plasmid DNA and vancomycin resistance in *Leuconostoc* spp. Appl. Environ. Microbiol. 48:1129-1133.

27. Piard, J.-C., P.M. Muriana, M. Desmazeaud, and T.R. Klaenhammer. 1990. Purification of lacticin 481, a bacteriocin produced by *Lactococcus lactis* CNRZ 481. FEMS Microbiol. Rev. 87:P90 (abstract).

28. Rammelsberg, M., E. Müller, and F. Radler. 1990. Caseicin 80: purification and characterization of a new bacteriocin from *Lactobacillus casei*. Arch. Microbiol. 154:249-252.

29. Roepstorff, P., P.F. Nielsen, I. Kamensky, A.G. Craig, and R. Self. 1988. ^{252}CF plasma desorption mass spectrometry of a polycyclic peptide antibiotic, nisin. Biomed. Environ. Mass Spectrom. 15:305-310.

30. Sahl, H.-G., and H. Brandis. 1981. Production, purification and chemical properties of an antistaphylococcal agent produced by *Staphylococcus epidermidis*. J. Gen. Microbiol. 127:377-384.

31. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain termination inhibitors. Proc. Nat'l. Acad. Sci. USA. 74:5463-5467.

32. Scopes, R. 1982. Protein Purification: Principles and Practice. Springer-Verlag, New York.

33. Tagg, J.R., A.S. Dajani, and L.W. Wannamaker. 1976. Bacteriocins of Gram-positive bacteria. Bacteriol. Rev. 40:722-756.

34. Wakamiya, T., K. Fukase, N. Naruse, M. Konishi, and T. Shiba. 1988. Lanthiopeptin, a new peptide effective against *Herpes simplex* virus: structural determination and comparison with Ro 09-0198, an immunopotentiating peptide. Tetrtahedron Lett. 29:4771-4772.

35. Wakamiya, T., T. Ueki, T. Shiba, Y. Kido, and Y. Motoki. 1985. The structure of ancovenin, a new peptide inhibitor of angiotensin I converting enzyme. Tetrahedron Lett. 26:665-668.

4. Cloning, Organization and Sequencing of a *Leuconostoc* Bacteriocin Operon[1]

4.1 Introduction

Recent interest in the antagonistic activity of lactic acid bacteria (LAB) has resulted in the cloning of several bacteriocin genes. The structural gene for nisin, a lantibiotic produced by *Lactococcus lactis* subsp. *lactis*, has been cloned and sequenced (4, 6, 13). Production of active nisin from a cloned piece of DNA has not yet been demonstrated. Analysis of the gene sequence revealed a 57-residue prepeptide that consists of a 23-residue leader peptide and a 34-residue bacteriocin that is posttranslationally modified to form non-ribosomally synthesized lanthionines and unsaturated amino acids (4). An insertion sequence IS904 is located upstream of the nisin determinant which appears to be a component of a large transmissible gene block that also encodes nisin resistance and sucrose metabolizing genes (6).

Two bacteriocin genes were cloned from a 60 kb plasmid resident in *L. lactis* subsp. *cremoris* (22). A 1.8 kb *Sca*I-*Cla*I fragment as well as a 1.3 kb *Sca*I-*Hin*dIII fragment encoding both production and immunity genes of different bacteriocins were cloned. On the 1.8 kb *Sca*I-*Cla*I fragment specifying low antagonistic activity, three open reading frames (ORF) were present and were organized in an operon. The first two ORFs encoded bacteriocin activity and contained 69 and 77 codons, respectively. The third ORF containing 154 codons was essential for immunity. Two ORFs were present on the 1.3 kb *Sca*I-*Hin*dIII fragment specifying high antagonistic activity. The first ORF containing 75 codons specified bacteriocin activity, whereas the second containing 98 codons specified immunity. There was homology between the nucleotide sequences of both fragments uptream of the first ORF and the first 20 bp of the first ORF of both bacteriocin operons

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were identical (22). Sherwitz-Harmon and McKay (21) cloned an 18 kb *Bcl*I fragment from an 88-MDa plasmid in *L. lactis* subsp. *lactis* WM4 that encoded bacteriocin production.

Recently, two structural genes encoding bacteriocins in lactobacilli have been reported. The structural gene encoding helveticin J produced by *Lactobacillus helveticus* 481 was cloned, sequenced and expressed in *Lactobacillus acidophilus* 88-C (12). Also, the structural gene encoding lactacin F produced by *Lactobacillus acidophilus* 11088 was cloned from a 110 kb plasmid using probe-hybridization. DNA sequence analysis elucidated a 75 amino acid precursor bacteriocin, consisting of a 57-residue bacteriocin and a 18-residue leader peptide (18). A gene encoding bacteriocin production and immunity was cloned from a 49 MDa plasmid (1) from *Carnobacterium piscicola* LV17 (Ahn and Stiles, personal communication).

The antimicrobial activities of *Leuconostoc gelidum* have been described (9, 10) and strain UAL 187 produces a small bacteriocin with a molecular weight of 3930.4 (10, 11). In this paper we report the cloning of the *Leucocin* A - UAL 187 gene and nucleotide sequence analysis. This is the first bacteriocin gene cloned from a *Leuconostoc* species.

4.2 Materials and methods

4.2.1 Bacterial strains, plasmids and media

The bacterial strains and plasmids used are listed in Table 4.1. *Escherichia coli* was grown in Luria broth or agar plates (1.5% agar) plus 20 mM glucose at 37°C. Selective concentrations of ampicillin and chloramphenicol for growing *E. coli* were 100 and 12.5 µg/ml, respectively. Carnobacteria, lactococci and leuconostocs were grown in APT broth or agar plates (1.5% agar) at 25°C. A selective concentration of 5.0 µg/ml chloramphenicol was used for carnobacteria and lactococci, and 2.5 µg/ml for leuconostocs.

Table 4.1 Bacterial strains and plasmids.

Bacterial Strain	Description	Source/Reference
Escherichia coli		
JM 103	*thr*, *rps*L, *end* A, *sbc*15, *hsd* R4, Δ (*lac pro* A, B), F' *tra* D36, *pro* AB, *lacI* ZΔM15	19
MV 1193	Δ (*lac-pro*AB), *rps*L, *thr*, *end* A, *spc*B15, *hsd* R4, Δ (*srl-rec*A) 306:: Tn 10 (*Tet*^{r}) F' [*tra* D36, *pro* AB$^+$, *lac* I^q *lac* Z Δ M15]	19
Leuconostoc gelidum		
UAL 187-22	Lcn$^+$ Imm$^+$ containing native plasmids, pLG 9.2 and pLG 7.6	10
UAL 187-13	Lcn$^-$ Imm$^-$ containing native plasmid, pLG 9.2	10
Carnobacterium piscicola UAL26	plasmidless	This laboratory
Leuconostoc sp. UAL60	plasmidless	This laboratory
Lactococcus lactis Na8	plasmidless	North Carolina State Univ.
PLASMIDS		
pUC 118	*lacZ'*, *Amp*^{r}, 3.2 kb	24
pNZ 19	*Cm*^{r}, *Km*^{r}, 5.7 kb *E. coli*/*Lactococcus* shuttle vector	Netherlands Inst. for Dairy Research
pJH 6.1F	*Amp*^{r}, *lcn* A, 6.1 kb	This study
pJH 8.6L	*Cm*^{r}, *Km*^{r}, *lcn* A, 8.6 kb	This study
pLG 9.2	Cryptic, 9.2 MDa	10
pLG 7.6	*lcn* A, 7.6 MDa	10

4.2.2 DNA isolation, manipulation and hybridization

Large scale plasmid preparations were done according to the method described by Anderson and McKay (2). Small-scale plasmid isolation from *E. coli* was done by the method described by Geneclean Bio 101 (LaJolla, Ca.). Restriction endonucleases and T4 DNA ligase were purchased from Boehringer-Mannheim (Dorval, Quebec) or Bethesda Research Laboratories (Burlington, Ontario) and used as recommended by the supplier. Procedures for DNA manipulations, cloning and hybridizations were done as described by Sambrook *et al.* (19). Competent cells of *E. coli* were transformed according to the procedure described by Chung *et al.* (5) or by electroporation according to procedures described by Luchansky *et al.* (16). Lactococci, carnobacteria and leuconostocs were transformed only by electroporation. Southern and colony blot hybridizations were done as described by Sambrook *et al.* (19). DNA was bound to Hybond N (Amersham Corp.) paper. Hybridizations and washes were done at 30°C. DNA probes were end-labelled with (gamma-^{32}P) dATP (Amersham Corp.) using T4 polynucleotide kinase (Pharmacia). A site specific 17-mer (JHA-3) (5' - CGAGAGCACTATTATCC - 3') was used to identify the *lcn* A gene in both colony and Southern hybridizations. Oligonucleotides were synthesized in the laboratory of Dr. K. Roy, Dept. of Microbiology, Univ. of Alberta on an Applied Biosystems 391 synthesizer PCR mate and used without further purification.

4.2.3 DNA sequencing analysis

Double stranded DNA was sequenced bidirectionally by the dideoxy chain termination method (20) by the DNA sequencing laboratory, Dept. of Biochemistry, Univ. of Alberta. Initial sequence was obtained by priming with a 24-mer wobbled oligonucleotide [5' - AAATATTATGG(TA)AATGG(TA)GT (XA)CAT - 3'][X= bromouracil] that corresponded to the degenerate N-terminal amino acid sequence of leucocin A-UAL 187 determined by Edman degradation (11; see Chapter 3). For further sequencing of both DNA strands, appropriate oligonucleotides were synthesized as previously described. Initially, DNA was sequenced using the 7.6 MDa plasmid of *L. gelidum* UAL 187-22 as

the template. After cloning the genes in pUC118, sequencing was continued using synthesized oligonucleotides from revealed sequence and M13/pUC multiple cloning site (MCS) universal forward and reverse primers. The DNA sequence data was submitted to GenBank (Los Alamos, NM) and the accession number M64371 was assigned to it. A search of the SWISPROT data bank using the EMBL implementation of the FASTA program by MAILFASTA service was done for the translation products of ORF's 1 and 2, by Dr. K. Roy, Dept. of Microbiology, Univ. of Alberta.

4.2.4 Molecular cloning of lcn*A gene*

Plasmid DNA from *L. gelidum* UAL 187-22 was digested with restriction enzymes compatible with the M13/pUC MCS. Southern blots of the restricted plasmid DNA revealed a 2.9 kb *Hpa*II fragment that hybridizes with the 17-mer JHA-3 deduced from known sequence within the *lcn* A gene. Plasmid DNA from a bacteriocin negative mutant, *L. gelidum* UAL 187-13 (10), was run in adjacent lanes as a control (Figure 4.1, lane A). The 2.9 kb *Hpa*II fragment was eluted from a gel using the Geneclean Bio 101 kit according to manufacturer's instructions, and ligated into the *Acc*I site of pUC118. The hybrid plasmid is referred to as pJH6.1F. Putative clones were confirmed by colony and Southern hybridization using primer JHA-3 as well as by restriction analysis of the insert and re-sequencing of regions already sequenced on the native plasmid pLG7.6.

4.2.5 Expression studies of leucocin in lactic acid bacteria

The 2.9 kb insert in pUC118 (pJH6.1F) was sub-cloned into the *PstI/SacI* sites of the shuttle vector, pNZ19, to make the hybrid pJH8.6L. The presence of the insert was confirmed by colony blot and Southern hybridization and by restriction analysis. Plasmid pJH8.6L was introduced into *Lactococcus lactis* Na8, *Leuconostoc sp.* UAL60, and *Carnobacterium piscicola* UAL26 by electroporation. The presence of pJH8.6L was confirmed by plasmid analysis, restriction analysis and Southern hybridization. Trans-

formants were confirmed by sugar fermentation testing. Bacteriocin production and immunity were tested by the deferred inhibition assay (10; see Chapter 2).

4.3 Results

4.3.1 Isolation of the leucocin structural gene, lcnA

Curing experiments indicated that the locus of the *lcn* A gene was situated on the 7.6 MDa plasmid of *Leuc. gelidum* UAL 187 (10). Plasmid DNA (pLG9.2 and pLG7.6) was isolated from *Leuc. gelidum* UAL 187-22 and digested with restriction enzymes compatible with the pUC118 MCS. A 17-mer deduced from the already known sequence of leucocin A-UAL 187 (previously determined by sequencing using the native plasmid pLG7.6 as a template) hybridized with a 2.9 kb *Hpa*II fragment (Figure 4.1, lane B). No signals were present from *Hpa*II restricted plasmid DNA from *Leuc. gelidum* UAL 187-13 containing only pLG 9.2 (Figure 4.1, lane A). This indicated that the *lcn*A structural gene was located on the 7.6 MDa plasmid and confirmed the previous curing experiments (10). Cloning of this 2.9 kb *Hpa*II fragment into the *Acc*I site of pUC118 resulted in a hybrid plasmid of 6.1 kb, pJH6.1F (Figure 4.1, lane C). Colony blot and Southern hybridizations done by probing with the sequencing primer JHA-3 , confirmed the presence of the *lcn* A gene (Figure 4.1, lane C).

4.3.2 Structure of the lcnA *gene*

DNA sequencing revealed two open reading frames (ORF) flanked by a putative promoter and terminator. According to the N-terminal amino acid sequence information of Leucocin A-UAL 187 (11), the leucocin peptide begins with lys 25 suggesting that the *lcn* A structural gene encodes a 61 amino acid prepeptide consisting of a 24 residue leader peptide and a 37 amino acid bacteriocin (Figure 4.2). A putative ribosomal binding site (RBS) GAGGA is located 9 bp upstream of the start codon (coordinate 293). A putative promotor (-35 and -10) is located 26 nucleotides upstream of the RBS (coordinate 261). A

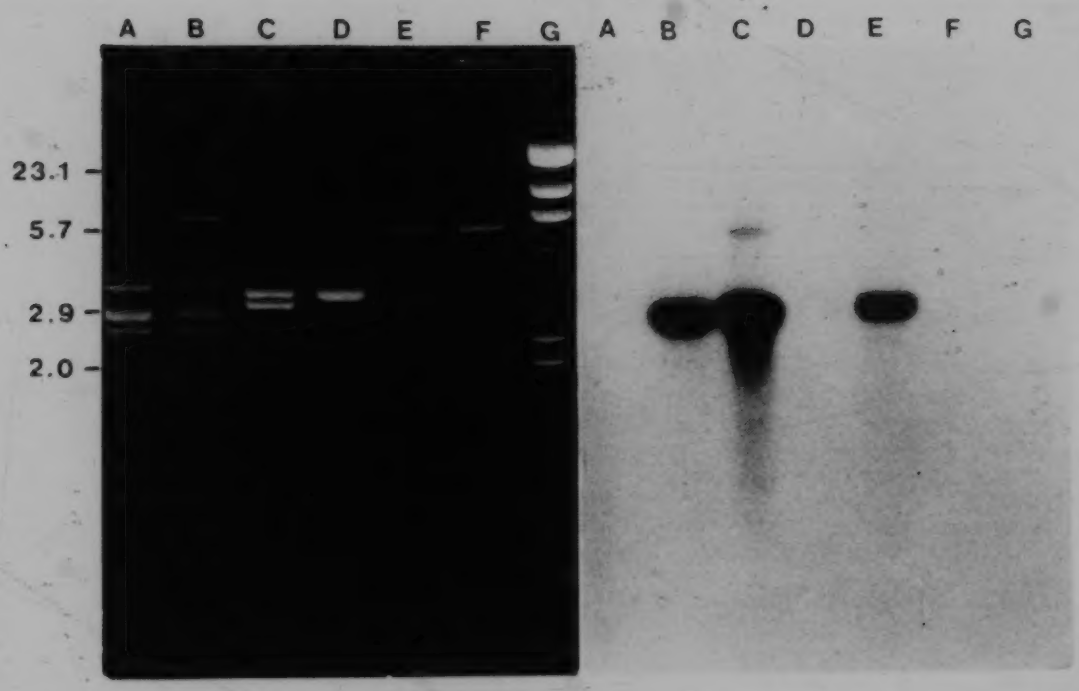

Figure 4.1 **Agarose gel electrophoresis and Southern blot probe hybridization of restriction digests of plasmids pLG9.2, pLG7.6, pJH6.1F and pJH8.6L** digested with restriction enzymes as follows: Lanes A, pLG9.6 *Hpa*II; B, pLG9.6 and pLG7.4 *Hpa*II; C, pJH6.1F *Sac*I/*Pst*I; D, pUC118 *Pst*I; E, pJH8.6F *Sac*I/*Pst*I; F, pNZ19 *Pst*I; and G, λ *Hind*III size standards (23.1, 9.4, 6.5, 4.4, 2.3, 2.0, and 0.56 kb). A site specific probe (JHA-3) homologous to a region within the leucocin structural gene was used in hybridization. The 2.9 kb *Hpa*II fragment of pLG7.6 (lane B) was cloned into pUC118 (lane C) and pNZ19 (lane E).

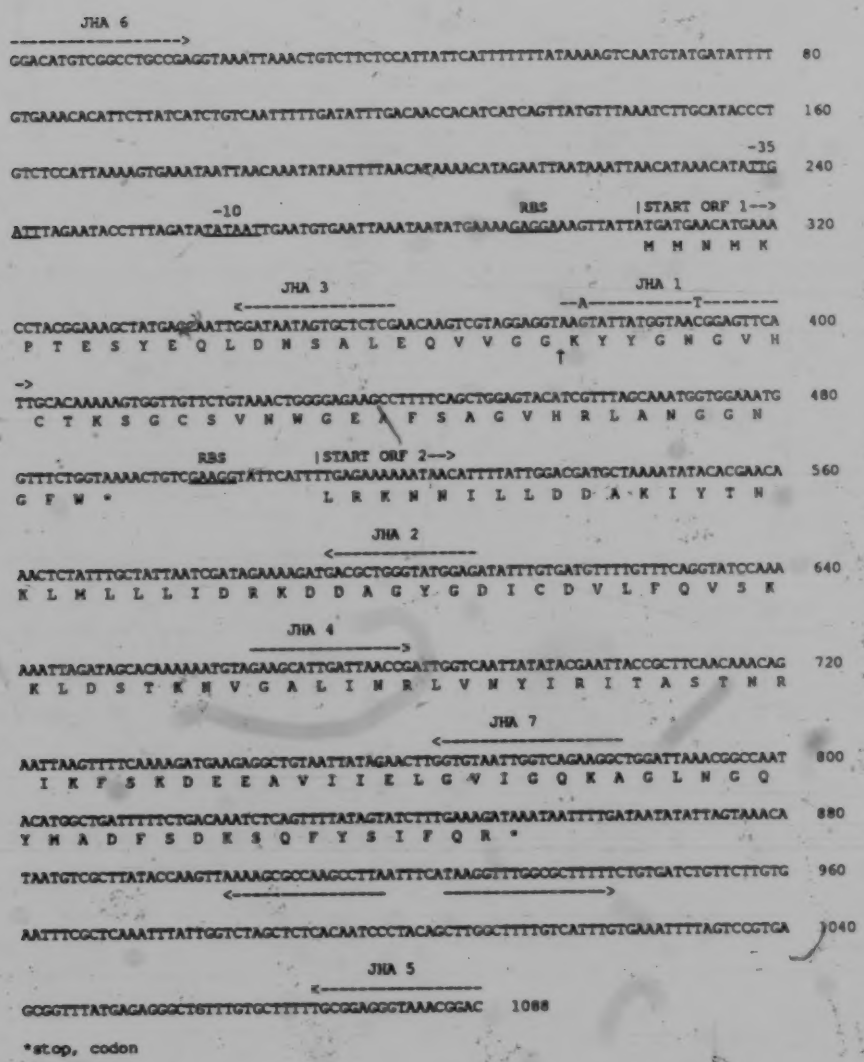

Figure 4.2 **Single-strand DNA sequence of the region of *Leuc. gelidum* UAL 187 plasmid (pLG7.6) containing the leucocin A operon.** ORF's 1 (precursor leucocin gene) and 2 (putative immunity protein) are indicated, with the translation products given below the nucleotide sequence. Probes/primers used in cloning and sequencing are shown by a dotted line above the nucleotides with mismatches indicated for the mixed oligomer JHA-1. The arrow between the glycine and lysine (coordinate 378) shows the point of cleavage of the N-terminal leader sequence. RBS, ribosomal binding site. The horizontal dashed arrows below the nucleotide sequence indicate inverted repeat sequences that are capable of forming stem-loop structures and may signal the termination of the transcript. Nucleotides between coordinates 64 to 1009 were confirmed by sequencing of both strands.

second ORF (ORF2) containing 113 amino acids was evident from the sequence (Figure 4.2). There is a putative RBS (GAAGG) 7 bp downstream of the ORF1 stop codon and 8 bp upstream of a TTG (leucine) codon which appears to be functioning as a start codon. A region of dyad symmetry that could form a stem-loop structure (Figure 4.3) was located downstream of ORF2 (coordinate 902, Figure 4.2). Sequencing with the reverse primer of the M13/pUC MCS displayed overlapping sequence at the 5' end of the leucocin operon indicating the position of the operon in the cloned insert (Figure 4.4). No protein in the SWISPROT data bank showed homology to the translation products of ORF's 1 and 2.

4.3.3 Studies on expression of the leucocin gene

The 2.9 kb insert containing the *lcn* A gene in pJH6.1F was removed by digestion with *Sac*I/*Pst*I and ligated into the equivalent sites in pNZ19 (5.7 kb). Chloramphenicol resistant *E. coli* JM103 colonies were screened by probe hybridization with primer JHA-3. Colonies that gave a positive signal showed the presence of an 8.6 kb plasmid that resolved into 5.7 kb and 2.9 kb fragments upon digestion with *Pst*I and *Sac*I. This new hybrid, pJH8.6L was successfully transferred into *Leuconostoc* sp. UAL 60 and *Lactococcus lactis* Na8, without apparent deletion of any part of the plasmid. The site specific primer JHA3 hybridized strongly to the plasmid pJH8.6L in both of these transformants. However, *C. piscicola* UAL 26 showed the presence of a smaller plasmid that may be a deletion derivative of pJH8.6L. No signal was evident upon hybridization with site specific primer JHA-3. None of the transformants showing the presence of the intact pJH8.6L or spontaneous deletion derivatives produced detectable levels of Leucocin A-UAL 187. We have been unable to transform a bacteriocin negative mutant of the original producer strain, *Leuc. gelidum* UAL 187-13, with the hybrid plasmid (pJH8.6L) nor with any other foreign plasmids.

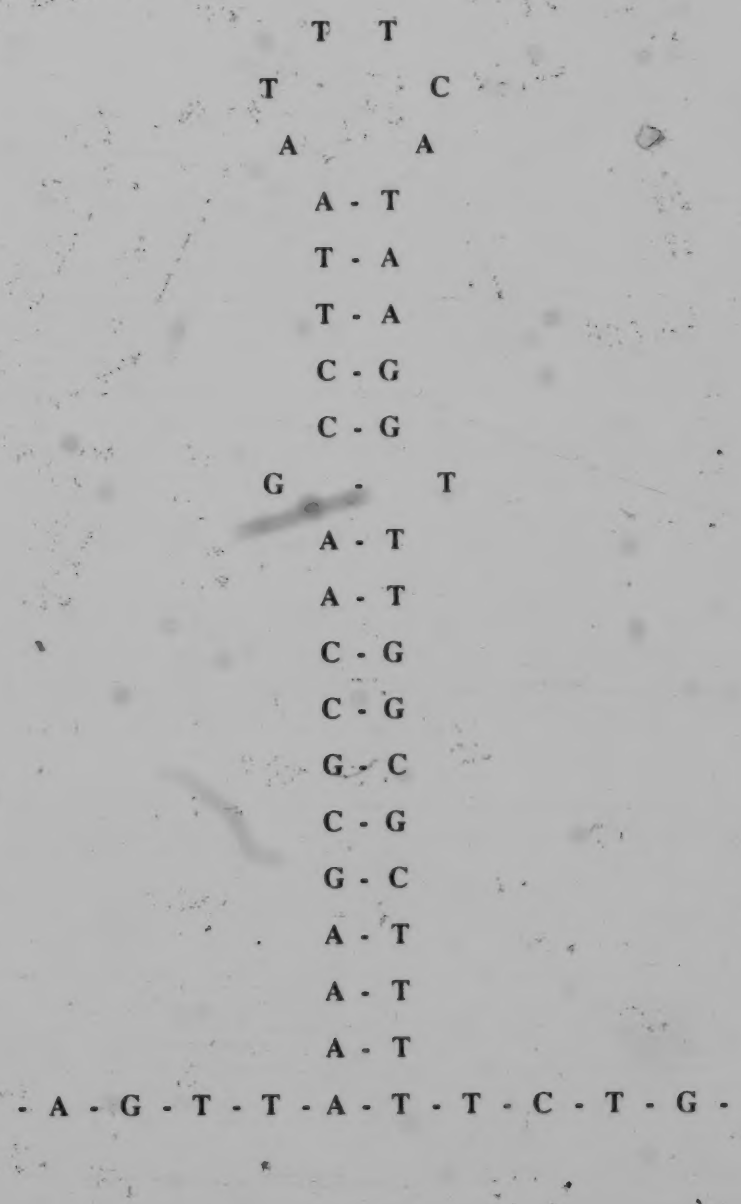

Figure 4.3 Region of dyad symmetry located 49 bp downstream of ORF 2 on the putative leucocin operon, that may form a stem loop structure and signal the termination of transcription.

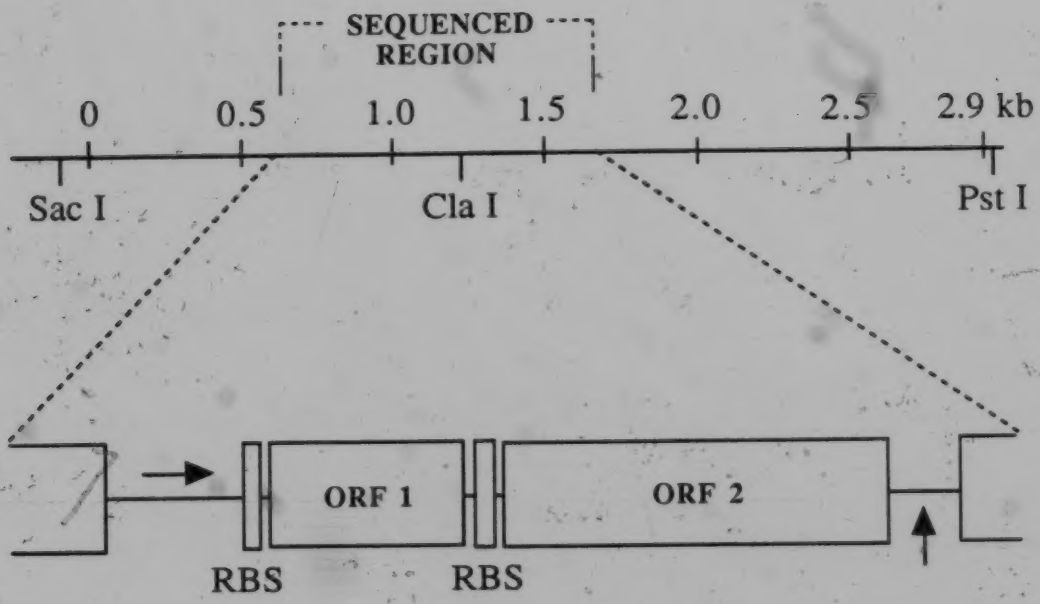

Figure 4.4 Location of ORF 1 (leucocin gene) and ORF 2 (putative immunity gene) in 2.9 kb fragment from *Leuconostoc gelidum* plasmid pLG7.6 cloned into the MCS of pUC118. *Pst*I and *Sac*I sites are part of the pUC118 MCS. Upper horizontal arrow, putative promoter; lower vertical arrow, putative terminator; RBS, ribosomal binding site.

4.3 Discussion

Leucocin A-UAL 187 is a small peptide (3930.4 daltons, 37 amino acids) and may be one of a group of small antibacterial peptides produced by the lactic acid bacteria. This group could include Lactacin F, 6.3 KDa, 57 amino acids (17, 18); Pediocin A, 2.7 KDa (3); and recently cloned bacteriocins from *L. lactis* which are encoded by genes containing, 69, 75 and 77 codons, part of which may be a leader peptide that is processed before secretion (22).

Knowing the first 12 amino acids of the N-terminus of leucocin A-UAL 187 allowed us to probe and immediately identify the ORF containing the *lcn* A structural gene and a probable 24-residue N-terminal extension. Initiation at the proposed Met is based on the distanee from the R.B.S. (Figure 4.2). This extension appears to be proteolytically cleaved before secretion, because the biologically active substance does not contain this region. This leader region may act as a signal sequence because it has a positively charged N-terminus, a typical non-charged C-region at the cleavage site and has some hydrophobicity in the core region (7). The 4 amino acids adjacent to the splice site in the C-region (V-V-G-G) are identical to those of the leader peptide of the *lac F* gene (18).

The bacteriocin structural gene may be transcribed from a putative promoter that is located upstream of the *lcn* A gene. The -10 region (TATAAT) and the first three nucleotides (TTG) of the -35 region follow a conserved pattern, but there is little consensus in the latter 3 nucleotides (14, 22, 23). The part of the gene coding for leucocin A-UAL 187 shows no DNA or amino acid homology to the Lactacin F gene or to the genes recently cloned from *L. lactis* subsp. *cremoris* (22). However, there is significant amino acid homology between *leucocin* A-UAL 187 and the bacteriocins produced by *Carnobacterium piscicola* UAL 8 (Ahn and Stiles, personal communication). The apparent operon consisting of a bacteriocin structural gene followed closely by a larger ORF, usually encoding immunity, seems to be a conserved pattern for this type of bacteriocin (18, 22). Further studies involving subcloning of ORF2 of this putative operon are required to establish the

function of this gene. The start codon of this gene is unusual. It is a TTG leucine rather than ATG methionine. In *E. coli*, GTG or TTG replace ATG as a start codon in about 9% of genes (8, 15). The frequency of this phenomenon in leuconostocs has not been determined. The putative terminator does not show a string of A's downstream of the stem-loop structure that is characteristic of a rho-independent terminator (25). Thus it may constitute a rho-dependent terminator or a splice site on a longer transcript as suggested for the nisin operon (4).

4.4.1 Expression studies

The inability of the lactic acid bacteria transformed with pJH8.6L to express leucocin production or immunity from the *lcn* A operon may be due to promoter recognition problems or the inability of the leader peptide to act as a transport protein in the hosts used or there may be a unique control mechanism operational, which is outside of the 2.9 kb cloned fragment. A further possibility would be the presence of a repressor gene within the 2.9 kb insert. Expression of the *lac F* (18) and bacteriocin genes from *L. lactis* subsp. *cremoris* (22) was successful from smaller cloned fragments of 2.2 kb and 1.8 kb, respectively. In both cases, production of and immunity to the bacteriocin were encoded by closely linked genes. We have not been successful in transforming a bacteriocin negative mutant of the parent strain. Until we are able to transform the native strain with the clone, we cannot determine whether or not we have sufficient genetic information for normal phenotypic expression of this bacteriocin. The position of the *lcn* A gene relative to the 2.9 kb insert and the genetic evidence of a putative promoter, immunity gene and termination signal all indicate that there should be sufficient information to obtain phenotypic expression in a suitable host.

4.5 Acknowledgement

The DNA sequencing was performed by the DNA Sequencing Laboratory, Department of Biochemistry, University of Alberta.

4.6 References

1. Ahn, C., and M.E. Stiles, 1990. Plasmid associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. Appl. Environ. Microbiol. 56:2503-2510.
2. Anderson, D.G., and L.L. McKay. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. 46:549-552.
3. Bhunia, A.K., M.C. Johnson, and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. J. Appl. Bacteriol. 65:261-268.
4. Buchman, G.W., S. Banerjee, and J.N. Hansen. 1988. Structure, expression and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. J. Biol. Chem. 263:16260-16266.
5. Chung, C.T., S.L. Niemela, and R.H. Miller. 1989. One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. 86:2172-2175.
6. Dodd, H.M., N. Horn, and M.J. Gasson. 1990. Analysis of the genetic determinant for production of the peptide antibiotic nisin. J. Gen. Microbiol. 136:555-566.
7. Gierasch, L.M. 1989. Signal sequences. Biochem. 28:923-930.
8. Gold, L.L., and G. Stormo. 1987. Translational initiation. In: *Escherichia coli* and *Salmonella typhimurium*., vol.2. F.C. Niedhardt (ed.). American Society for Microbiology. Washington, D.C. pp. 1302-1307.
9. Harding, C.D., and B.G. Shaw. 1990. Antimicrobial activity of *Leuconostoc gelidum* against closely related species and *Listeria monocytogenes*. J. Appl. Bacteriol. 69:648-654.
10. Hastings, J.W., and M.E. Stiles. 1991. Antibiosis of *Leuconostoc gelidum* isolated from meat. J. Appl. Bacteriol. 70:127-134.

11. Hastings, J.W., M. Sailer, K. Johnson, J.C. Vederas, and M.E. Stiles. 1991. Purification and characterization of leucocin A-UAL 187, a bacteriocin from *Leuconostoc gelidum*. J. Bacteriol. (submitted).

12. Joerger, M.C., and T.R. Klaenhammer. 1990. Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. J. Bacteriol. 172:6339-6347.

13. Kaletta, C., and K.D. Entian. 1989. Nisin, a peptide antibiotic: Cloning and sequencing of the *nis*A gene and post-translational processing of its peptide product. J. Bacteriol. 17:1594-1601.

14. Koivula, T., M. Sibakov, and I. Palva. 1991. Isolation and characterization of *Lactococcus lactis* subsp. *lactis* promoters. Appl. Environ. Microbiol. 57:333-340.

15. Kozak, M. 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol. Rev. 47:1-45.

16. Luchansky, J.B., P.M. Muriana, and T.R. Klaenhammer. 1988. Electrotransformation of Gram-positive bacteria. Bulletin 1350. Biorad Laboratories, Mississauga, Ontario.

17. Muriana, P.M., and T.R. Klaenhammer. 1991. Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. Appl. Environ. Microbiol. 57:114-121.

18. Muriana, P.M., and T.R. Klaenhammer. 1991. Cloning, phenotypic expression and DNA sequence of the gene for Lactacin F, an antimicrobial peptide produced by *Lactobacillus* spp. J. Bacteriol. 173:1779-1788.

19. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

20. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463-5467.

21. Scherwitz Harmon, K., and L.L. McKay. 1987. Restriction enzyme analysis of lactose and bacteriocin plasmids from *Streptococcus lactis* subsp. *diacetylactis* WM_4 and cloning of *Bcl* I fragments coding for bacteriocin production. Appl. Environ. Microbiol. 53:1171-1174.

22. Van Belkum, M.J., B.J. Hayema, R.E. Jeeninga, J. Kok, and G. Venema. 1991. Organization and nucleotide sequences of two lactococcal bacteriocin operons. Appl. Environ. Microbiol. 57:492-498.

23. Van der Vossen, J.M.B.M., D. van der Lelie, and G. Venema. 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. 53:2452-2457.

24. Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3.

25. Yager, T.D., and P.H. von Hippel. 1987. Transcript elongation and termination in *Escherichia coli*. In: *Escherichia coli* and *Salmonella typhimurium*., vol.2. F.C. Niedhardt (ed.). American Society for Microbiology, Washington. D.C. pp. 1241-1275.

5. General Conclusions

The three major objectives of this research were: (1) to characterize a lactic acid bacterium that was producing an inhibitory substance and to determine the physiological characteristics of its production; (2) to develop an efficient method to isolate and purify the antagonistic substance, in order to obtain sufficient yields for detailed characterization; and (3) to characterize the inhibitory substance both by isolation and examination of its genetic determinants and by biochemical analysis of the pure substance. The overall purpose of obtaining this information is to understand the mechanism whereby these inhibitory substances are produced and to elucidate the relationship of the structure of the substance to its function as an inhibitor of bacteria.

5.1 Taxonomic identity of the producer strain and physiological characteristics of the production of the inhibitory substance

The first task of positively identifying this organism was of importance primarily because of the possibility of studying an already known bacteriocin. Recently, an inhibitory substance thought to be novel and produced by *Lactobacillus plantarum* was later revealed to be the already well characterized bacteriocin, nisin (4). The problem occurred because of the insufficient characterization of the producer strain. A thorough examination of the strain showed that it was, in fact, *Lactococcus lactis*, and that the bacteriocin being produced was nisin. Initial experiments in this study revealed the producer strain to be *Leuconostoc gelidum*, a new species of a genera that had not been studied with respect to bacteriocin production and characterization. The initial studies also showed that this organism produces an inhibitor that is active against a wide range of lactic acid bacteria (LAB) and is most active against the important food pathogen, *Listeria monocytogenes*. Thus this substance has the potential to assist a producr strain to dominate in a mixed fermentation of lactic acid bacteria or to act as a preservative agent against growth of pathogenic organisms.

The physiological production characteristics were appealing in that the bacteriocin is produced over a wide range of pH and temperature conditions, creating the potential for its

application to a broad range of foods and food storage conditions. The production of the bacteriocin early in the growth cycle and throughout the growth cycle may also be an important factor in enabling a producer strain to dominate a microbial population in a food.

5.2 Development of an efficient purification method for small antimicrobial peptides

Historically, research on the purification of bacteriocins of the LAB has resulted in considerable losses of activity and quantity of protein (1, 2, 3, 5, 6, 7). Essentially, the meager amounts of peptide available at the end of the process prohibited further characterization. A relatively simple isolation procedure with good yields was developed in this study. Subsequently, our laboratory has been successful in purifying and isolating at least four other bacteriocins using the same method, with slight modifications. This procedure may, therefore, have a broad applicability to the efficient isolation of small hydrophobic bacteriocins.

The success of this isolation procedure may be attributable to: (1) establishing that these compounds are most stable at acidic pH, and maintaining low pH throughout the procedure was crucial to the stability of this compound during isolation; (2) the avoidance of dialysis which caused at least 50% loss of activity; and (3) using hydrophobic interaction chromatography which resulted in almost 100% recovery of bacteriocin compared with losses of greater than 90% when ion exchange chromatography was used. The first fermentation and subsequent isolation yielded greater than 2 mg of protein, which exceeds previous yields by a factor of 20 to 200 (1, 6). This enabled further characterization of the compound. Determination of the N-terminal amino acid sequence, the amino acid content, the exact molecular weight of the compound by mass spectrometry and some stability characteristics of the purified bacteriocin were all accomplished from the products of the first fermentation. The information obtained concerning the exact molecular weight and the existence of secondary structure in the peptide is unique in the field of non-lantibiotic bacteriocins produced by lactic acid bacteria.

Therefore, the purification and characterization studies of leucocin A-UAL 187 are significant because the yield of bacteriocin obtained in a pure form is significantly higher than previously achieved, and the degree of characterization is greater than any other non-lantibiotic bacteriocin from lactic acid bacteria (1, 2, 3, 5, 6, 7).

5.3 Characterization of genetic determinants

Prior determination of the amino acid sequence of the N-terminus of leucocin A-UAL 187 enabled the synthesis of an oligonucleotide probe which was used to locate and clone the leucocin operon, as well as to begin sequencing in the area of the gene. The subsequent genetic characterization of the clone revealed an intact operon that should be capable of expressing the bacteriocin in a suitable host strain. Current experiments involving electroporation of dextranase- and lysozyme-treated cells are being conducted to try to transform a bacteriocin negative mutant of the parent strain, *Leuconostoc gelidum* UAL 187-13, with foreign plasmids. Alternatively, expression cassette polymerase chain reaction technology (8) could be used to express large quantities of leucocin in a suitable strain. When an efficient expression system is achieved, experiments involving manipulation of the gene could be performed to improve production quantities or to extend the spectrum of activity of this bacteriocin. Our laboratory has isolated several other bacteriocins from a different strain of lactic acid bacteria. Some appear to be very similar to leucocin A-UAL 187, yet they have different specifities in terms of antagonistic spectra. This indicates that changing a few amino acids may significantly affect the activity spectrum of the bacteriocin. Site directed mutagenesis studies could confirm this.

The bacteriocin, leucocin A-UAL 187, may not be the "super bacteriocin" that is desired for application to meats (or foods). However, it has served as a prototype and has "paved the way" for further research by establishing guidelines for isolation, purification and biochemical and genetic characterization of similar small antimicrobial peptides. These

"new" compounds may be found by further screening, or manufactured by genetic modification of an existing well characterized bacteriocin such as leucocin A-UAL 187.

5.4 References

1. Barefoot, S.F., and T.R. Klaenhammer. 1984. Purification and characterization of the *Lactobacillus acidophilus* bacteriocin Lactacin B. Antimicrob. Agents Chemother. 26:328-334.
2. Bhunia, A.K., M.C. Johnson, and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. J. Appl. Bacteriol. 65:261-268.
3. Joerger, M.C., and T.R. Klaenhammer. 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Bacteriol. 167:439-446.
4. Klaenhammer. T.R. 1988. Bacteriocins of lactic acid bacteria. Biochimie 70:337-349.
5. Mortvedt, C.I., J. Nissen-Meyer, and I.F. Nes. 1990. Purification of a bacteriocin from *Lactobacillus sake* and its association with a 50 kb plasmid. FEMS Microbiol. Rev. 87:P90 (abstract)
6. Muriana, P.M., and T.R. Klaenhammer. 1991. Purification and partial characterization of Lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. Appl Environ. Microbiol. 57:114-121.
7. Rammelsberg, M., E. Muller, and F. Radler. 1990. Caseicin 80: Purification and characterization of a new bacteriocin from *Lactobacillus casei*. Arch. Microbiol. 154:249-252.
8. Schreiber, S.L., and G.L. Verdine. 1991. Protein overproduction for organic chemists. Tetrahedron 47:2543-2562.